08/663618

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No: 27866/32960

PATENT APPLICATION TRANSMITTAL

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith for filing is the patent application of

Inventor(s): Patrick W. Gray

Title: Chitinase Materials and Methods

1. Type of Application

This new application is for a

☒ utility patent.

☐ design patent.

2. Application Papers Enclosed

1	Title Page
47	Pages of Specification (excluding Claims, Abstract & Drawings)
3	Page(s) of Claims
1	Page(s) of Abstract
0	Sheet(s) of Drawings (Figs. ___ to ___)
	Formal
	Informal

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this Patent Application Transmittal and the documents referred to as enclosed therewith are being deposited with the United States Postal Service on **June 14, 1996**, in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 utilizing the "Express Mail Post Office to Addressee" service of the United States Postal Service under Mailing Label No. EM118658816US.

Li-Hsien Rin-Laures

Li-Hsien Rin-Laures

3. **Declaration or Oath**

☐ Enclosed

☐ Executed by (check all applicable boxes)

☐ Inventor(s)

☐ Legal representative of inventor(s) (37 CFR 1.42 or 1.43)

☐ Joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached

☐ The petition required by 37 CFR 1.47 and the statement required by 37 CFR 1.47 are enclosed. See Item 5D below for fee.

☒ Not enclosed - the undersigned attorney or agent is authorized to file this application on behalf of the applicant(s). An executed declaration will follow.

4. **Additional Papers Enclosed**

☐ Preliminary Amendment

☐ Information Disclosure Statement

☐ Declaration of Biological Deposit

☒ Computer readable copy of sequence listing containing nucleotide and/or amino acid sequence and statement under 37 C.F.R. §1.821

☐ Verified statement(s) claiming small entity status under 37 CFR 1.9 and 1.27

☐ Associate Power of Attorney

☐ Verified translation of a non-English patent application

☐ An assignment of the invention

☐ Certified copy(ies) of application(s):

COUNTRY	APPLICATION NO.	FILED

from which priority under 35 USC 119 is claimed ☐ is(are) attached.

☐ will follow.

☐ Other

5. **Filing Fee Calculation (37 CFR 1.16)**

A. ☒ **Utility Application**

CLAIMS AS FILED - INCLUDING PRELIMINARY AMENDMENT (IF ANY)						
			SMALL ENTITY		OTHER THAN A SMALL ENTITY	
	NO. FILED	NO. EXTRA	RATE	FEE	RATE	FEE
BASIC FEE				$375.00		$750.00
TOTAL	31 -20	= 11	X 11 =	$	X 22 =	$242.00
INDEP.	14 - 3	= 11	X 39 =	$	X 78 =	$858.00
☒ First Presentation of Multiple Dependent Claim			+ 125 =	$	+ 250 =	$250.00
Filing Fee:				$	**OR**	$2100.00

B. ☐ **Design Application ($155.00/$310.00)** Filing Fee: $ _____

C. ☐ **Plant Application ($255.00/$510.00)** Filing Fee: $ _____

D. **Other Fees**

☐ Recording Assignment [Fee -- **$40.00** per assignment] $ _____

☐ Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached [Fee -- **$130.00**] $ _____

☐ Other $ _____

Total Fees Enclosed **$2100.00**

6. **Method of Payment of Fees**

☒ Enclosed check in the amount of: $2100.00

☐ Charge Deposit Account No. 13-2855 in the amount of: $_____
A copy of this Transmittal is enclosed.

☐ Not enclosed

7. Deposit Account and Refund Authorization

The Commissioner is hereby authorized to charge any deficiency in the amount enclosed or any additional fees which may be required during the pendency of this application under 37 CFR 1.16 or 37 CFR 1.17 or under other applicable rules (except payment of issue fees), to Deposit Account No. 13-2855. A copy of this Transmittal is enclosed.

Please refund any overpayment to Marshall, O'Toole, Gerstein, Murray & Borun at the address below.

Please direct all future communications to Li-Hsien Rin-Laures, at the address below.

Respectfully submitted,

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By: Li Hsien Rin-Laures
Li-Hsien Rin-Laures
Reg. No: 33,547

June 14, 1996

PATENT
DOCKET NO. 27866/32960

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

Application of:

Patrick W. Gray

Serial No: 08/663,618

Filed: June 14, 1996

For: Chitinase Materials and Methods

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail, postage prepaid, in an envelope addressed to: Assistant Commissioner for Patents Washington, D.C. 20231, on this date

August 26, 1996

Li Hsien Rin-Laures

Li-Hsien Rin-Laures

STATEMENT UNDER 37 C.F.R. §1.825(d)

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I hereby declare under 36 C.F.R. §1.825(d) that the contents of the substitute computer readable form of the Sequence Listing submitted herewith are the same as the contents of the paper copy of the Sequence Listing as originally filed pursuant to 37 C.F.R. §1.821(c).

Respectfully submitted,

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By: Li Hsien Rin-Laures

Li-Hsien Rin-Laures
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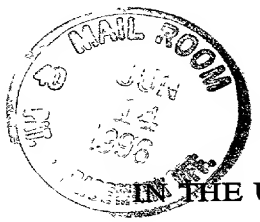
August 26, 1996



PATENT APPLICATION
ATTORNEY DOCKET NO. 27866/32960

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:

Patrick W. Gray

Serial No:

Filed: HEREWITH

For: CHITINASE MATERIALS AND METHODS

"EXPRESS MAIL" mailing label No. EM118658816US

Date of Deposit: June 14, 1996

I hereby certify that this paper (or fee) is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents Washington, DC 20231

Li-Hsien Rin-Laures

Li-Hsien Rin-Laures, M.D.
(Reg. No. 33,547)
Attorney for Applicant

STATEMENT UNDER 37 C.F.R. §1.821(f)

Assistant Commissioner for Patents
Washington, DC 20231

Sirs:

I hereby state that the content of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 C.F.R. §1.821(c) and (e), respectively, are the same.

Respectfully submitted,

MARSHALL, O'TOOLE, GERSTEIN,
MURRAY & BORUN

BY: Li-Hsien Rin-Laures

Li-Hsien Rin-Laures
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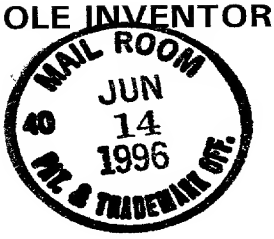
Chicago, Illinois
June 14, 1996

SOLE INVENTOR



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Li-Hsien Rin-Laures

Li-Hsien Rin-Laures

APPLICATION FOR UNITED STATES LETTERS PATENT

SPECIFICATION

TO ALL WHOM IT MAY CONCERN:

Be it known that I, Patrick W. Gray a citizen of United States, residing at 2244 38th Place East, Seattle 98112, in the County of King and State of Washington, have invented a new and useful CHITINASE MATERIALS AND METHODS, of which the following is a specification.

CHITINASE MATERIALS AND METHODS

FIELD OF THE INVENTION

The present invention relates generally to human chitinase enzyme and more specifically to novel purified and isolated polynucleotides encoding human chitinase, to the chitinase products encoded by the polynucleotides, to materials and methods for the recombinant production of chitinase products and to antibody substances specific for the chitinase.

BACKGROUND

Chitin is a linear homopolymer of β-(1,4)-linked N-acetylglucosamine residues. This polysaccharide is second only to cellulose as the most abundant organic substance. The exoskeleton of arthropods is composed of chitin. In addition, fungi and other parasites contain chitin in their outer cell wall, where it serves important structural and protective roles. Disruption of the fungal cell wall and membrane has been a useful therapeutic strategy against fungi and parasites. For example, Amphotericin B and fluconazole exert their anti-fungal activity by affecting membrane steroids. Despite the existence of anti-fungal therapeutics, fungal infections of humans have increasingly become responsible for life-threatening disorders. See, Georgopapadakou *et al.*, *Trends Microbiol.*, *3:* 98-104 (1995). The fungal species and parasites responsible for these diseases are mainly *Candida, Aspergillus, Cryptococcus, Histoplasma, Coccidioides* and *Pneumocystis*. These pathogens are particularly dangerous in immunocompromised individuals, such as patients with AIDS, patients undergoing chemotherapy, and immunosuppressed organ transplant patients.

Chitin can be degraded by the enzyme chitinase. Chitinase enzymes are found in plants, microorganisms, and animals. Bacterial chitinase helps to provide a carbon source for bacterial growth. Insects produce chitinase to digest their cuticle at each molt. In plants, chitinase is thought to provide a protective role against parasitic fungi. Chitinases have been cloned from numerous bacterial [*e.g.*, *Serratia marcescens*, Jones et al., *EMBO J.*, *5*:467-473 (1986)], plant [*e.g.*, tobacco,

Heitz et al., *Mol. Gen. Genet., 245*:246-254 (1994)], and insect [*e.g.*, wasp, Krishnan et al., *J. Biol. Chem., 269*:20971-20976 (1994)] species.

Several proteins with low homology to bacterial, insect, and plant chitinases (less than 40% amino acid identity) have been identified in mammals, such as human cartilage gp-39 (C-gp39) [Hakala *et al., J. Biol. Chem., 268:*25803-25810 (1993)], human glycoprotein YKL-40 [Johansen et al., *Eur. J. Cancer, 31A*:1437-1442 (1995)], oviduct-specific, estrogen-induced protein from sheep [DeSouza *et al., Endocrinology, 136:*2485-2496 (1995)], cows and humans; and a secretory protein from activated mouse macrophages [Chang *et al.*, Genbank M94584]. However, chitin-degrading activity has not been reported for these proteins. The function of these proteins is not known, but they have been postulated to be involved in tissue remodeling. Hakala et al., *supra*, report that C-gp39 is detectable in synovial and cartilage specimens from rheumatoid arthritis patients, but not from normal humans. Recklies et al., *Arthritis Rheumatism, 36*(9 SUPPL.):S190 (1993) report localization of the C-gp39 protein to a distinct population of cells in the superficial layers of cartilage. Johansen et al., *supra*, report that measurements of YKL-40 serum levels are of value as a potential prognostic marker for the extent of metastatic disease and survival of patients with recurrent breast cancer.

Escott et al., *Infect. Immun., 63*:4770-4773 (1995) demonstrated chitinase enzymatic activity in human leukocytes and in human serum. Overdijk et al., *Glycobiology, 4*:797-803 (1994) described isolation of a chitinase (4-methylumbelliferyl-tetra-N-acetylchitotetraoside hydrolase) from human serum and rat liver. Renkema *et al., J. Biol. Chem., 270:*2198-2202 (February 1995) prepared a human chitotriosidase from the spleen of a Gaucher disease patient. Their preparation exhibited chitinase activity and the article reports a small amount of amino acid sequence of the protein component of the preparation (22 amino terminal residues and 21 residues of a tryptic fragment). The function of human chitinase is also unknown, but a relationship with the pathophysiology of Gaucher disease is proposed in the article. A later publication by the same group [Boot *et al., J. Biol. Chem., 270*(44):26252-26256 (November 1995)] describes the cloning of a human macrophage cDNA encoding a product that exhibits chitinase activity. The partial

amino acid sequence reported by the group in their February 1995 article matches portions of the deduced amino acid sequence of the human macrophage cDNA product.

In view of the increasing incidence of life-threatening fungal infection in immunocompromised individuals, there exists a need in the art to identify and isolate polynucleotide sequences encoding human chitinases, to develop materials and methods useful for the recombinant production of the enzyme, and to generate reagents for the detection of the chitinase in plasma.

SUMMARY OF THE INVENTION

The present invention provides novel purified and isolated polynucleotides (*i.e.*, DNA and RNA, both sense and antisense strands) encoding human chitinase or fragments and analogs thereof; methods for the recombinant production of chitinase polypeptides, fragments and analogs thereof; purified and isolated chitinase polypeptide fragments and analogs; antibodies to such polypeptides, fragments and analogs; and pharmaceutical compositions comprising these polypeptides, fragments, analogs, or antibodies.

Specifically provided are: purified, isolated polynucleotides encoding the human chitinase amino acid sequence of SEQ ID NOS: 2 or 4, particularly amino acids 1 to 445 thereof; DNAs comprising the protein coding nucleotides of SEQ ID NOS: 1 or 3, particularly nucleotides 65 to 1402 of SEQ ID NO: 1 or nucleotides 90 to 1427 of SEQ ID NO: 3; purified, isolated polynucleotides comprising a polynucleotide sequence encoding the amino acid sequence of SEQ ID NOS: 14 or 15; purified, isolated polynucleotides encoding human chitinase selected from the group consisting of: (a) a double-stranded DNA comprising the protein coding portions of the sequence set out in SEQ ID NO: 1, (b) a DNA which hybridizes under stringent conditions to a non-coding strand of the DNA of (a), and (c) a DNA which, but for the redundancy of the genetic code, would hybridize under stringent conditions to a non-coding strand of DNA sequence of (a) or (b); vectors comprising such DNAs, particularly expression vectors wherein the DNA is operatively linked to an expression control DNA sequence; host cells stably transformed or transfected with

such DNAs in a manner allowing the expression in said host cell of human chitinase; a method for producing human chitinase comprising culturing such host cells in a nutrient medium and isolating human chitinase from said host cell or said nutrient medium; purified, isolated polypeptides produced by this method; purified, isolated polypeptides comprising the human chitinase amino acid sequence of SEQ ID NOS: 2 or 4, particularly amino acids 1 to 445 thereof; human chitinase fragments lacking from 1 to about 72 C-terminal amino acid residues of mature human chitinase, particularly the human chitinase fragment of SEQ ID NO: 14; the human chitinase analog of SEQ ID NO: 15; hybridoma cell lines producing a monoclonal antibody that is specifically reactive with one of the above-described polypeptides; and monoclonal antibodies produced by such hybridomas.

Preferred DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences. The nucleotide sequence of two human cDNAs encoding presumed allelic variants of human chitinase, and including noncoding 5' and 3' sequences, are set forth in SEQ ID NO: 1 and SEQ ID NO: 3. These DNA sequences and DNA sequences which hybridize to the noncoding strand thereof under standard stringent conditions or which would hybridize but for the redundancy of the genetic code, are contemplated by the invention. Preferred DNAs of the present invention comprise the human chitinase coding region (corresponding to nucleotides 2 to 1402 of SEQ ID NO: 1 or nucleotides 27 to 1427 of SEQ ID NO: 3), and the putative coding sequence of the mature, secreted human chitinase protein without its signal sequence (nucleotides 65 to 1402 of SEQ ID NO: 1, or nucleotides 90 to 1427 of SEQ ID NO: 3).

Exemplary stringent hybridization conditions are as follows: hybridization at 42°C in 50% formamide and washing at 60°C in 0.1 x SSC, 0.1% SDS. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide base content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact hybridization conditions. See Sambrook *et al.*, 9.47-9.51 in *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

Two amino acid sequences for human chitinase(s) are set forth in SEQ ID NOS: 2 and 4. The sequence of SEQ ID NO: 2 is encoded by the nucleotide sequence of SEQ ID NO: 1, and SEQ ID NO: 4 is encoded by the nucleotide sequence of SEQ ID NO: 3. Preferred polynucleotides of the present invention include, in addition to those polynucleotides described above, polynucleotides that encode amino acids -21 to 445 of SEQ ID NO: 2 or SEQ ID NO: 4, and that differ from the polynucleotides described in the preceding paragraphs only due to the well-known degeneracy of the genetic code. Similarly, since twenty-one amino acids (positions -21 to -1) of SEQ ID NOS: 2 and 4 comprise a signal peptide that is cleaved to yield the mature human chitinase protein, preferred polynucleotides include those encoding polypeptides comprising amino acids 1 to 445 of SEQ ID NO: 2 or SEQ ID NO: 4.

Among the uses for the polynucleotides of the present invention is use as a hybridization probe, to identify and isolate genomic DNA encoding human chitinase; to identify and isolate non-human genes encoding proteins homologous to human chitinase; to identify human and non-human proteins having similarity to human chitinase (including those that may be involved in tissue remodeling); and to identify those cells which express human chitinase and the biological conditions under which this protein is expressed.

In another aspect, the invention includes biological replicas (i.e., copies of isolated DNA sequences made *in vivo* or *in vitro*) of DNA sequences of the invention. Autonomously replicating recombinant constructions such as plasmid and viral DNA vectors incorporating chitinase polynucleotides, including any of the DNAs described above, are provided. Preferred vectors include expression vectors in which the incorporated chitinase-encoding cDNA is operatively linked to an endogenous or heterologous expression control sequence and a transcription terminator. Such expression vectors may further include polypeptide-encoding DNA sequences operably linked to the chitinase-encoding DNA sequences, which vectors may be expressed to yield a fusion protein comprising the polypeptide of interest.

According to another aspect of the invention, procaryotic or eucaryotic host cells are stably transformed or transfected with DNA sequences of the invention

in a manner allowing the desired chitinase product to be expressed therein. Host cells expressing chitinase products can serve a variety of useful purposes. Such cells constitute a valuable source of immunogen for the development of antibody substances specifically immunoreactive with chitinase. Host cells of the invention are useful in methods for the large scale production of chitinase wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated, *e.g.*, by immunoaffinity purification, from the cells or from the medium in which the cells are grown.

Chitinase products may be obtained as isolates from natural cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving procaryotic or eucaryotic host cells of the invention. Chitinase products of the invention may be full length polypeptides, fragments or analogs thereof. Chitinase products having part or all of the amino acid sequence set out in SEQ ID NO: 2 or SEQ ID NO: 4 are contemplated. One preferred fragment which lacks the C-terminal seventy-two amino acid residues of the mature protein is set forth in SEQ ID NO: 14. It has been determined that these seventy-two C-terminal residues are not critical to chitinase enzymatic activity. Example 5 illustrates production of this C-terminal fragment; the introduction of a stop codon after the codon for amino acid 373 resulted in a recombinant chitinase fragment of about 39 kDa that retained similar specific activity when compared with full length recombinant human chitinase.

Analogs may comprise chitinase analogs wherein one or more of the specified (i.e., naturally encoded) amino acids is deleted or replaced or wherein one or more nonspecified amino acids are added: (1) without loss of one or more of the enzymatic activities or immunological characteristics specific to chitinase; or (2) with specific disablement of a particular biological activity of chitinase. Example 3 illustrates the production of such an analog (SEQ ID NO: 15), in which the proline at position 370 is substituted with a serine, and in which the C-terminal seventy-two amino acid residues have been deleted. The use of mammalian host cells is also expected to provide for post-translational modifications (*e.g.*, myristolation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation)

as may be needed to confer optimal biological activity on recombinant expression products of the invention.

Proteins or other molecules that bind to chitinase may be used to modulate its activity. Also comprehended by the present invention are antibody substances (e.g., monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, CDR-grafted antibodies and the like) and other binding proteins specific for chitinase. Proteins or other molecules (*e.g.*, small molecules) which specifically bind to chitinase can be identified using chitinase isolated from plasma, recombinant chitinase, chitinase variants or cells expressing such products. Binding proteins are useful, in turn, in compositions for immunization as well as for purifying chitinase, and are useful for detection or quantification of chitinase in fluid and tissue samples by known immunological procedures. Anti-idiotypic antibodies specific for chitinase-specific antibody substances are also contemplated.

The scientific value of the information contributed through the disclosures of DNA and amino acid sequences of the present invention is manifest. As one series of examples, knowledge of the sequence of a cDNA for chitinase makes possible the isolation by DNA/DNA hybridization of genomic DNA sequences encoding chitinase and chitinase expression control regulatory sequences such as promoters, operators and the like. DNA/DNA hybridization procedures carried out with DNA sequences of the invention under conditions of stringency standard in the art are likewise expected to allow the isolation of DNAs encoding human allelic variants of chitinase, other structurally related human proteins sharing one or more of the biochemical and/or immunological properties of chitinase, and non-human species proteins homologous to chitinase. The DNA sequence information provided by the present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, *e.g.*, Kapecchi, *Science, 244*: 1288-1292 (1989)], of rodents that fail to express a functional chitinase enzyme, overexpress chitinase enzyme, or express a variant chitinase enzyme. Polynucleotides of the invention when suitably labelled are useful in hybridization assays to detect the capacity of cells to synthesize chitinase. Polynucleotides of the invention may also be the basis for diagnostic methods useful for identifying a genetic alteration(s) in the chitinase locus

that underlies a disease state or states. Also made available by the invention are anti-sense polynucleotides relevant to regulating expression of chitinase by those cells which ordinarily express the same.

Administration of chitinase preparations of the invention to mammalian subjects, especially humans, for the purpose of ameliorating disease states caused by chitin-containing parasites such as fungi is contemplated by the invention. Pathogenic fungi cause serious, often fatal disease in immunocompromised hosts. Cancer patients undergoing chemotherapy, immunosuppressed individuals, and HIV-infected individuals are susceptible to mycoses caused by *Candida*, *Aspergillus*, *Pneumocystis carinii*, and other fungi. Amphotericin B and fluconazole are useful therapeutics for fungal infections, but toxicity associated with these drugs causes serious adverse side effects that limit their usefulness. The mortality of systemic candidiasis is greater than 50% despite Amphotericin B treatment. Therefore, a need exists for agents that inhibit fungal growth *in vivo*; and such products may be used as single agents or in combination with currently approved, conventional anti-fungal compounds. Because growing fungi require chitin synthesis for survival, inhibition by recombinant human chitinase may be useful for limiting fungal infections *in vivo*. Animal models for fungal infection are illustrated below in Examples 8 through 14 and have been described in the art.

Specifically contemplated by the invention are chitinase compositions for use in methods for treating a mammal susceptible to or suffering from fungal infections comprising administering chitinase to the mammal in an amount sufficient to supplement endogenous chitinase activity. It is contemplated that the chitinase may be administered with other conventional anti-fungal agents, including amphotericin B and the structurally related compounds nystatin and pimaricin; 5-fluorocytosine; azole derivatives such as fluconazole, ketoconazole, clotrimazole, miconazole, econazole, butoconazole, oxiconazole, sulconazole, terconazole, itraconazole and tioconazole; allylamines-thiocarbamates, such as tolnaftate, naftifine and terbinafine; griseofulvin; ciclopirox olamine; haloprogin; undecylenic acid; and benzoic acid. [See, *e.g.*, Goodman & Gilman, The Pharmacological Basis of Therapeutics, 9th ed., McGraw-Hill, NY (1996).] Chitinase may improve the effectiveness of these conventional

anti-fungal agents, perhaps by rendering the yeast more susceptible to their action, even in situations where the chitinase alone is not effective for inhibiting growth of fungi. By reducing the amount of conventional anti-fungal agent needed to exert the desired therapeutic effect, chitinase may allow the drugs to be used at less toxic levels.

Therapeutic/pharmaceutical compositions contemplated by the invention include chitinase and a physiologically acceptable diluent or carrier and may also include other anti-fungal agents. Dosage amounts indicated would be sufficient to supplement endogenous chitinase activity. For general dosage considerations see *Remington: The Science and Practice of Pharmacy*, 19th ed., Mack Publishing Co., Easton, PA (1995). Dosages will vary between about 1 μg/kg to 100 mg/kg body weight, and preferably between about 0.1 to about 20 mg chitinase/kg body weight. Therapeutic compositions of the invention may be administered by various routes depending on the infection to be treated, including via subcutaneous, intramuscular, intravenous, intrapulmonary, transdermal, intrathecal, topical, oral, or suppository administration.

The invention also contemplates that the overexpression of chitinase in Gaucher disease or at sites of inflammation (such as in rheumatoid arthritis) may have deleterious effects on the extracellular matrix and, in such disease settings, inhibitors of chitinase activity may provide therapeutic benefit, *e.g.* by reducing remodeling or destruction of the extracellular matrix.

The human chitinase CDNA of the present invention was isolated from a macrophage cDNA library. Macrophages are known to be closely associated with rheumatoid arthritis lesions [Feldman et al., *Cell, 85*:307-310 (1996)], and macrophage products such as TNF-α are implicated in disease progression. A protein with homology to human chitinase, C-gp39, has been detected in the synovium and cartilage of rheumatoid arthritis patients. While the natural substrate for human chitinase is probably chitin from pathogenic organisms, the enzyme may also exhibit activity on endogenous macromolecules which form the natural extracellular matrix. For example, it has been suggested that hyaluronic acid, a major component of the extracellular matrix, contains a core of chitin oligomers. [Semino *et al., Proc. Nat'l*

Acad. Sci., *93*:4548-4553 (1996); Varki, *Proc. Nat'l. Acad. Sci.*, *93*:4523-4525 (1996).] Chitinase may therefore be involved in degradation of extracellular matrix in diseases such as rheumatoid arthritis. The role of chitinase may be determined by measuring chitinase levels and/or the effects of chitinase administration or chitinase inhibition in synovial fluid isolated from arthritic joints. Endogenous chitinase levels can be measured by enzymatic assay or with an antibody. Viscosity of synovial fluid can be measured before and after chitinase treatment; a decrease of viscosity associated with chitinase would be consistent with an endogenous chitinase substrate. Modulation of chitinase activity could thereby modulate the progression of joint destruction in rheumatoid arthritis.

Also contemplated by the invention are methods for screening for inhibitors of chitinase activity, which may be useful in the manner described in the preceding paragraph. A method for screening samples to identify agents that inhibit chitinase is reported in, *e.g.*, WO 95/34678 published 21 December 1995.

Further contemplated are methods for measuring endogenous levels of chitinase, *e.g.*, for diagnosing Gaucher's disease. Hollak et al., *J. Clin. Invest.*, *93*:1288-1292 (1994), report that plasma chitinase levels are a diagnostic marker for Gaucher's disease. The recombinant proteins of this invention are expected to be more useful than preparations purified from humans, which have associated problems of yield and contamination with other impurities or infectious agents.

DETAILED DESCRIPTION

Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. Example 1 describes the isolation of human chitinase cDNA clones from a human macrophage cDNA library. Example 2 addresses the pattern of chitinase gene expression in various human tissues. Example 3 describes the recombinant expression of the human chitinase gene in prokaryotic cells and purification of the resulting enzyme. Example 4 provides a protocol for the recombinant production of human chitinase in yeast. Example 5 describes the recombinant expression of the human chitinase gene in mammalian cells and purification of the resulting protein. Example 6 describes

production of human chitinase polypeptide analogs by peptide synthesis or recombinant production methods. Example 7 provides a protocol for generating monoclonal antibodies that are specifically immunoreactive with human chitinase. Example 8 describes an assay for the measurement of chitinase catalytic activity. Example 9 addresses determination of the anti-fungal activity of human chitinase *in vitro*. Example 10 addresses determination of the anti-fungal activity of human chitinase *in vivo* in a mouse model, and Examples 11 through 14 address rabbit models of invasive aspergillosis, disseminated candidiasis, *Candida* ophthalmitis, and *Candida* endocarditis.

Example 1

Isolation of Chitinase cDNA Clones

A cDNA library was prepared from peripheral blood monocyte-derived macrophages as described in Tjoelker et al., *Nature*, 374:549-552 (1995). Clones from the library were randomly chosen and plasmid DNA was purified from individual clones. The sequence of approximately 300 to 500 bases from the end of DNA from each clone was determined on an automated sequencer (Model 373, Applied Biosystems, Foster City, CA) using primer JHSP6, which hybridizes to the plasmid vector pRc/CMV (Invitrogen, San Diego, CA) adjacent to the cDNA cloning site:

JHSP6: 5'-GACACTATAGAATAGGGC-3' (SEQ ID NO: 5)

The nucleotide and deduced amino acid sequence of these cDNA clones were compared to sequences in nucleotide and peptide sequence databases to determine similarity to known genes. Sequence comparisons were performed by the BLAST Network Service of the National Center for Biotechnology Information using the alignment algorithm of Altschul et al., *J. Mol. Biol.*, *215*:403-410 (1990). Clone MO-911 exhibited significant homology to several different sequences, including mouse macrophage secretory protein YM-1 precursor (Genbank accession no. M94584), human cartilage gp-39 (Hakala *et al.*, *supra*), oviductal glycoprotein from sheep, cow, and humans (DeSouza *et al.*, *supra*), and chitinases from parasite (Oncocerca, Genbank accession no. U14639), wasp (*Chelonus*, Genbank accession no.

U10422), plant (*Nicotiana*, Genbank accession no. X77111), and bacteria (*Serratia*, Genbank accession no. Z36295); its highest observed homology was to mammalian genes that encoded proteins with chitinase homology but no demonstrated chitinase activity. Further sequence analysis of MO-911 suggested that it contained a portion of the coding region for a human chitinase homolog.

The DNA sequence of clone pMO-218 (deposited on June 7, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. under Accession No. 98077) is set forth in SEQ ID NO: 1, and the encoded amino acid sequence is set forth in SEQ ID NO: 2. MO-218 appeared to include the entire coding region of the human chitinase cDNA (nucleotides 2 to 1402 of SEQ ID NO: 1), which comprises a twenty-one amino acid putative signal sequence followed by 445 encoded amino acids (residues 1 to 445 of SEQ ID NO: 2). The twenty-two amino acids following the putative signal sequence exactly match the amino-terminal sequence of purified human chitotriosidase reported in Renkema *et al.*, *supra*. Renkema *et al.* also described a twenty-one amino acid sequence from a tryptic fragment of human chitotriosidase which corresponds exactly to residues 157 to 177 of MO-218 (SEQ ID NO: 2). Boot et al., *supra*, report the cloning of a human chitotriosidase cDNA which contains a coding sequence essentially identical to that of MO-218. The sequence of MO-218 differs from Boot et al. by an additional fourteen nucleotides at the 5' end and by a nucleotide change at nucleotide 330 in the coding region.

To confirm that MO-218 indeed contained the entire coding region of the cDNA, a ^{32}P-labelled probe P-1 (TGGGATCATCAGCAGGACCATGAAACCTGCCCAGGCCACAGACCGCACCAT, SEQ ID NO: 6) was prepared that corresponded to the complement of nucleotides 2 through 52 of MO-218 (SEQ ID NO: 1). Probe P-1 was designed to hybridize with clones that are at least as long as MO-218 at the 5' end. The probe was hybridized with a portion (approximately 30,000 clones) of the human macrophage cDNA library described above, in 40% formamide and hybridization buffer (5 x SSPE, 10 x Denhardt's, 100 μg/ml denatured salmon sperm DNA, and 2% SDS) at 42°C overnight. The filters were washed and three clones that hybridized were chosen for

sequence /analysis. The longest clone was designated pMO-13B (deposited on June 7, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. under Accession No. 98078). The DNA sequence of pMO-13B is set forth in SEQ ID NO: 3 and the encoded amino acid sequence is set forth in SEQ ID NO: 4. This clone contains 25 additional nucleotides at the 5' end compared with MO-218; in addition, MO-13B (SEQ ID NO: 3) contains one nucleotide substitution at nucleotide 330 (corresponding to nucleotide 305 of MO-218, SEQ ID NO: 1) which changes the encoded amino acid at position 80 of the mature protein from a glycine (in SEQ ID NO: 2) to a serine (in SEQ ID NO: 4).

Example 2

Chitinase Gene Expression Pattern in Human Tissues

Northern blot analysis was performed to identify tissues in which the human chitinase is expressed. A multiple human tissue Northern blot (Clontech, Palo Alto CA) was hybridized with the entire coding region of MO-218 under standard stringent conditions (according to the Clontech laboratory manual). Greatest hybridization was observed to lung tissue (+++) and ovary (+++), with much smaller levels (+) in thymus and placenta. The size of the hybridizing mRNA was 2.0 kb for lung, ovary and thymus, which corresponds well with the size of the cloned cDNA (1.6 kb, or about 1.8 kb including the polyA tail). The size of the hybridizing placental mRNA was considerably smaller, at 1.3 kb. Chitinase hybridization was not observed in spleen, prostate, testes, small intestine, colon, peripheral blood leukocytes, heart, brain, liver, skeletal muscle, kidney, or pancreas. Chitinase expression in lung is consistent with a protective role against pathogenic organisms that contain chitin, since the lung represents the primary route of entry for fungal pathogens.

Example 3

Production of Recombinant Human Chitinase in Bacterial Cells

The mature coding region of MO-218 was engineered for expression in *E. coli* as a C-terminal truncated analog. PCR was used to generate a DNA fragment for expression using a primer corresponding to nucleotides 65 to 88 of the MO-218 chitinase cDNA preceded by an initiating methionine codon and an XbaI restriction endonuclease site (5'-TACATCTAGAATTATGGCAAAACTGGTCTGCTACTTCACC-3', SEQ ID NO: 7), and a downstream primer encoding nucleotides 1163 to 1183 of MO-218 followed by a stop codon and a HindIII site (5'-AGATCTAACCTTAGGTGCCTGAAGACAAGTATGG-3', SEQ ID NO: 8). The downstream primer contained an adenine at base 25, while the MO-218 sequence contains a guanine at the corresponding nucleotide position. Consequently, the resulting DNA fragment contains a thymine rather than a cytosine at the position corresponding to nucleotide 1172 of the MO-218 sequence, and the encoded chitinase fragment, set forth in SEQ ID NO: 15, is also an analog that contains a serine at mature amino acid position 370 instead of the proline encoded by MO-218. The resulting DNA fragment was digested with XbaI and HindIII and cloned into plasmid pAraBAD (which is also known by the designation pAraCB).

Plasmid pAraCB was prepared as follows. Plasmid pUC19 was modified to include an arabinose promoter and subsequently to include AKAP 79 encoding sequences. The arabinose promoter [Wilcox *et al.*, *Gene* **34**:123-128 (1985); Wilcox, *et al.*, *Gene* **18**:157-163 (1982)] and the araC gene were amplified by PCR from the arabinose operon BAD of *Salmonella typhimurium* as an *Eco*RI/*Xba*I fragment with the primers araC-2 (SEQ ID NO: 9) and arab-1 (SEQ ID NO: 10):

araC-2 TACAGAATTC*TTA*TTCACATCCGGCCCTG SEQ ID NO: 9

arab-1 TACATCTAGA*CTCC*ATCCAGAAAAACAGGTATGG SEQ ID NO: 10

Primer araC-2 encodes an *Eco*RI site (underlined) and a termination codon (italics) for the araC gene product. Primer arab-1 encodes a putative ribosome binding domain

(italics) and an *Xba*I restriction site (underlined). PCR with these primers produced a 1.2 kb fragment which was digested with *Eco*RI and *Xba*I and subcloned into pUC19 (New England Biolabs, Beverly, MA) previously digested with the same two enzymes. The resulting plasmid was designated araCB and contained a polylinker region (SEQ ID NO: 11) flanked at the 5´ end with a *Xba*I restriction site (underlined) and at the 3´ end with a *Hin*dIII site (italics).

araCB polylinker SEQ ID NO: 11

TCTAGAGTCGACCTGCAGGCATGC*AAGCTT*

Transformants containing the resulting expression plasmid (pAraMO218) were induced with arabinose and grown at 37°C. These transformants produced inclusion bodies containing a 39 kDa protein which was a truncated form of chitinase (engineered to contain 373 instead of 445 amino acids). This chitinase fragment contains four cysteine residues, while the full length chitinase contains ten cysteine residues. The inclusion bodies were separated from the *E. coli* culture and electrophoresed on SDS-PAGE. The 39 kDa band was transferred to a PVDF membrane and amino terminal sequenced. The majority (about two-thirds) of the material contained a sequence corresponding to the amino terminus of human chitinase. The remaining material corresponded to a contaminating *E. coli* protein, porin. This recombinant chitinase preparation from *E. coli* was useful for producing polyclonal and monoclonal antibodies (described below in Example 7).

When transformants containing the Ara-chitinase expression plasmid were grown at 25°C, inclusion bodies were not observed and expression of recombinant product was decreased from about ten percent of total cell protein to about one percent. However, this material produced at 25°C exhibited chitinase catalytic activity.

Example 4

Production of Recombinant Human Chitinase in Yeast Cells

Exemplary protocols for the recombinant expression of human chitinase in yeast and for the purification of the resulting recombinant protein follow. The coding region of human chitinase is engineered into vectors for expression in *Saccharomyces cerevisiae* using either PCR or linker oligonucleotides designed to encode a fusion polypeptide containing a secretion mediating leader to the coding region for human chitinase corresponding to the amino terminus of the natural molecule. Secretion signal peptides include, *e.g.*, SUC2 or equivalent leaders with a functional signal peptidase cleavage site, or pre-pro-alpha factor or other complex leader composed of a pre, or signal peptide, and a pro, or spacer region, exhibiting a KEX2 cleavage site. The DNA encoding the signal sequence can be obtained by oligonucleotide synthesis or by PCR. The DNA encoding the pre-pro-alpha factor leader is obtained by PCR using primers containing nucleotides 1 through 20 of the alpha mating factor gene and a primer complementary to nucleotides 255 through 235 of this gene [Kurjan and Herskowitz, *Cell, 30:*933-943 (1982)]. The pre-pro-alpha leader coding sequence and human chitinase coding sequence fragments are ligated into a plasmid containing the yeast alcohol dehydrogenase (ADH2) promoter, such that the promoter directs the expression of a fusion protein. As taught by Rose and Broach, [*Meth. Enz., 185*:234-279, D. Goeddel, ed., Academic Press, Inc., San Diego, CA (1990)], the vector further includes an ADH2 transcription terminator downstream of the cloning site, the yeast "2-micron" replication origin, a selectable marker, for example TRP1, CUP1 or LEU2 (or LEU2-d) or other equivalent gene, the yeast REP1 and REP2 genes, the *E. coli* beta lactamase gene, and an *E. coli*

origin of replication. The beta-lactamase and TRP1 genes provide for selection in bacteria and yeast, respectively. The REP1 and REP2 genes encode proteins involved in plasmid copy number replication.

Alternatively, other fusion points within the chitinase coding region may be chosen. Truncates of the coding region may be used to increase homogeneity of the product, increase the specific activity or alter the substrate specificity.

The DNA constructs described in the preceding paragraphs are transformed into yeast cells using a known method, *e.g.* lithium acetate treatment [Stearns *et al., Meth. Enz., supra*, pp. 280-297] or by equivalent methods. The ADH2 promoter is induced upon exhaustion of glucose in the growth media [Price *et al., Gene, 55*:287 (1987)]. The pre-pro-alpha sequence or other leader sequence effects secretion of the fusion protein, releasing the mature human chitinase peptide from the cells. The signal peptide leader is processed by signal peptidase or, in the case of pre-pro-alpha removal of the pro region, by the KEX2 protease [Bitter *et al., Proc. Natl. Acad. Sci. USA, 81*:5330-5334 (1984)].

Chitinase contains in its mature amino acid sequence two dibasic sequences at positions 107-108 (Lys-Arg) and 209-210 (Arg-Lys) that may be proteolytically clipped by the KEX2 protease during secretion. To stabilize and/or increase the level of product secreted from cells, these sequences could be mutated to eliminate the potential sites for proteolysis as shown by Gillis *et al. [Behring Inst. Mitt., No. 83*:1-7 (1988)] or by expressing chitinase without dibasic modifications in a host that is deficient in KEX2. Such hosts can be obtained either by screening for non-KEX2 protease containing mutants, or by manipulation of the genomic KEX2

locus by gene replacement/gene disruption techniques [Orr-Weaver *et al.*, *Proc. Natl. Acad. Sci, USA*, *78*:6354-6358 (1981)].

Recombinant chitinase may be secreted from *Saccharomyces cerevisiae* using similar vectors containing alternative promoters PRB1, GAL4, TPI, or other suitably strong promoters bearing fragments or by fusion to a variety of leader sequences [Sleep *et al.*, *Bio/Technol.*, *8*:42-46 (1990)].

Other non-*Saccharomyces cerevisiae* suitable expression hosts include *Kluyveromyces lactis, Schizosaccharomyces pombe, Pichia pastoris* and members of the *Hansenula* or *Aspergillus* geni. Analogous recombinant expression systems for these fungi include the organism and their appropriate autonomously replicating vector [*e.g.* Falcone *et al.*, *Plasmid*, *15*:248-252 (1988)] or multiply integrated expression cassettes. These systems also rely on signal sequences or leaders of the types described above to mediate secretion into the medium.

The secreted recombinant human chitinase is purified from the yeast growth medium by, *e.g.*, the methods used to purify chitinase from bacterial and mammalian cell supernatants (see Example 3 above and Example 5 below).

Alternatively, the mature form of the recombinant chitinase product may be expressed in the cytoplasms of the *Saccharomyces cerevisiae* cells or analogous host, and purified from the lysed host cells. The protein may be refolded during the act of purification to obtain appropriate levels of specific activity.

Example 5

Production of Recombinant Human Chitinase in Mammalian cells

The MO-218 clone and the MO-13B clone, both of which contain full length human chitinase cDNA 3' to the CMV promoter of pRc/CMV, were isolated. A third plasmid, which corresponded to the same C-terminal fragment expressed in bacterial cells in Example 3 above, was prepared as follows. The MO-218 plasmid was amplified by PCR using oligonucleotide primer 218-1 (CGCAAGCTTGAGAGCTCCGTTCCGCCACATGGTGCGGTCTGTGGCCTGGG, SEQ ID NO: 12), which contains a Hind III site and nucleotides 2 through 23 of the MO-218 chitinase cDNA of SEQ ID NO: 1, and with complementary downstream primer T-END (GACTCTAGACTAGGTGCCTGAAGGCAAGTATG, SEQ ID NO: 13), which contains nucleotides 1164 through 1183 of MO-218, a stop codon and an XbaI site. The amplified DNA was purified by electrophoresis, digested with XbaI and HindIII, and cloned into the pRc/CMV vector (Invitrogen, San Diego, CA) previously cut with the same restriction enzymes. The junctions of the resulting clone was sequenced on a Model 373 (Applied Biosystems, Foster City, CA) and encoded the predicted engineered protein sequence, set forth in SEQ ID NO: 14.

All three plasmids were transiently transfected into COS cells by the DEAE transfection method [see, *e.g.*, Sambrook *et al.*, *Molecular Cloning: a Laboratory Manual*, 2d ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989).). After three days at 37°C, media from cells was assayed for chitinase activity *in vitro* as described below in Example 8. Each culture produced significant chitinase activity (600-800 mU/ml/min), and similar amounts were observed for each construct.

Recombinant human chitinase was purified as follows. Five days after transfection of COS cells with the pRc/CMV-MO-13B plasmid, conditioned media from the culture was harvested and diluted with an equal volume of water. The diluted conditioned media was applied to a Q-Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) which was pre-equilibrated in 25 mM Tris, 10 mM sodium chloride, 1 mM EDTA, at pH 8.0. Approximately 95% of the chitinase activity flowed through and did not bind to the column. This Q-Sepharose flow through was adjusted to 1.2 M ammonium sulfate and applied to a Butyl-Sepharose 4 Fast Flow column (Pharmacia) which was pre-equilibrated in 25 mM Tris, 1.2 M ammonium sulfate, 1 mM EDTA, at pH 8.0. Protein was eluted using a reverse gradient of 1.2 M to 0 M ammonium sulfate in 25 mM Tris, pH 8.0. A single absorbance peak at 280nm corresponding to the chitinase activity peak was eluted at low salt. This material was greater than 85% pure as determined by SDS-PAGE and contained approximately 60% of the chitinase activity. The protein was then concentrated and buffer exchanged into 20 mM Tris, 150 mM sodium chloride, at pH 8.0 using a 10,000 MWCO membrane (Ultrafree 10K, Millipore Corp., Bedford, MA). This preparation was then tested for enzymatic and anti-fungal activity *in vitro* as described in Examples 8 and 9 below. The recombinant preparation had a chitotriosidase activity of 90 nm/min per mg protein.

Example 6

Production of Human Chitinase Analogs and Fragments

Recombinant techniques such as those described in the preceding examples may be used to prepare human chitinase polypeptide analogs or fragments. More particularly, polynucleotides encoding human chitinase are modified to encode polypeptide analogs of interest using well-known techniques, *e.g.*, site-directed mutagenesis and polymerase chain reaction. C-terminal and N-terminal deletions may also be prepared by, *e.g.*, deleting the appropriate portion of the polynucleotide coding sequence. See generally Sambrook *et al.*, *supra*, Chapter 15. The modified polynucleotides are expressed recombinantly, and the recombinant polypeptide analogs or fragments are purified as described in the preceding examples.

Residues critical for human chitinase activity are identified, *e.g.*, by homology to other chitinases and by substituting alanines for the native human chitinase amino acid residues. Cysteines are often critical for the functional integrity of proteins because of their capacity to form disulfide bonds and restrict secondary structure. To determine whether any of the cysteines in human chitinase are critical for enzymatic activity, each cysteine is mutated individually to a serine.

A 39 kDa C-terminally truncated fragment of the mature human chitinase protein was prepared as described above in Examples 3 and 5 by introduction of a stop codon after the codon for amino acid 373. This 39 kDa fragment lacked seventy-two C-terminal amino acid residues of the mature protein, including six cysteines, yet retained similar specific enzymatic activity compared to the full length recombinant human chitinase. This result indicates that the missing

seventy-two C-terminal residues, including the six cysteines, are not required for enzymatic activity.

Further C-terminal deletions may be prepared, *e.g.*, by digesting the 3' end of the truncated human chitinase coding sequence described in Example 3 with exonuclease III for various amounts of time and then ligating the shortened coding sequence to plasmid DNA encoding stop codons in all three reading frames. N-terminal deletions are prepared in a similar manner by digesting the 5' end of the coding sequence and then ligating the digested fragments into a plasmid containing a promoter sequence and an initiating methionine immediately upstream of the promoter site. These N-terminal deletion analogs or fragments may also be expressed as fusion proteins.

Alternatively, human chitinase polypeptide analogs may also be prepared by full or partial chemical peptide synthesis using techniques known in the art. [*See, e.g.*, synthesis of IL-8 in Clark-Lewis *et al.*, *J. Biol Chem.*, *266*:23128-34 (1991); synthesis of IL-3 in Clarke-Lewis *et al.*, *Science, 231*:134-139 (1986); and synthesis by ligation in Dawson et al., *Science, 266*:776-779 (1994).] Such synthetic methods also allow the selective introduction of novel, unnatural amino acids and other chemical modifications.

The biological activities, including enzymatic, anti-fungal, and extracellular matrix remodeling activities, of the human chitinase polypeptide analogs are assayed by art-recognized techniques, such as those described in Examples 8 to 14 below.

Example 7

Preparation of Monoclonal Antibodies to Human Chitinase

The following two protocols (multiple challenge or single shot immunizations) may be used to generate monoclonal antibodies to human chitinase. In the first protocol, a mouse is injected periodically with recombinant human chitinase (*e.g.*, 10-20 μg emulsified in Freund's Complete Adjuvant) obtained as described in any of Examples 3 through 6. The mouse is given a final pre-fusion boost of human chitinase in PBS, and four days later the mouse is sacrificed and its spleen removed. The spleen is placed in 10 ml serum-free RPMI 1640, and a single cell suspension is formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum-free RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension is filtered through sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, New Jersey), and is washed twice by centrifuging at 200 g for 5 minutes and resuspending the pellet in 20 ml serum-free RPMI. Splenocytes taken from three naive Balb/c mice are prepared in a similar manner and used as a control. NS-1 myeloma cells, kept in log phase in RPMI with 11% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah) for three days prior to fusion, are centrifuged at 200 g for 5 minutes, and the pellet is washed twice as described in the foregoing paragraph.

One x 10^8 spleen cells are combined with 2.0 x 10^7 NS-1 cells and centrifuged, and the supernatant is aspirated. The cell pellet is dislodged by tapping the tube, and 1 ml of 37°C PEG 1500 (50% in 75mM Hepes, pH 8.0) (Boehringer Mannheim) is added with stirring over the course of 1 minute, followed by the

addition of 7 ml of serum-free RPMI over 7 minutes. An additional 8 ml RPMI is added and the cells are centrifuged at 200 g for 10 minutes. After discarding the supernatant, the pellet is resuspended in 200 ml RPMI containing 15% FBS, 100 μM sodium hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine (HAT) (Gibco), 25 units/ml IL-6 (Boehringer Mannheim) and 1.5 x 10^6 splenocytes/ml and plated into 10 Corning flat-bottom 96-well tissue culture plates (Corning, Corning New York).

On days 2, 4, and 6, after the fusion, 100 μl of medium is removed from the wells of the fusion plates and replaced with fresh medium. On day 8, the fusion is screened by ELISA, testing for the presence of mouse IgG binding to human chitinase as follows. Immulon 4 plates (Dynatech, Cambridge, MA) are coated for 2 hours at 37°C with 100 ng/well of human chitinase diluted in 25mM Tris, pH 7.5. The coating solution is aspirated and 200 ul/well of blocking solution [0.5% fish skin gelatin (Sigma) diluted in CMF-PBS] is added and incubated for 30 min. at 37°C. Plates are washed three times with PBS with 0.05% Tween 20 (PBST) and 50 μl culture supernatant is added. After incubation at 37°C for 30 minutes, and washing as above, 50 μl of horseradish peroxidase conjugated goat anti-mouse IgG(fc) (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted 1:3500 in PBST is added. Plates are incubated as above, washed four times with PBST, and 100 μL substrate, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 μl/ml 30% H_2O_2 in 100 mM Citrate, pH 4.5, are added. The color reaction is stopped after 5 minutes with the addition of 50 μl of 15% H_2SO_4. A_{490} is read on a plate reader (Dynatech). Selected fusion wells are cloned twice by dilution into 96-well plates and visual scoring of the number of colonies/well after 5 days. The monoclonal

antibodies produced by hybridomas are isotyped using the Isostrip system (Boehringer Mannheim, Indianapolis, IN).

Alternatively, a second protocol utilizing a single-shot intrasplenic immunization may be conducted generally according to Spitz, *Methods Enzymol.*, *121*:33-41 (1986). The spleen of the animal is exposed and injected with recombinant human chitinase (*e.g.*, 10-20 μg in PBS at a concentration of about 0.02% to 0.04%, with or without an aluminum adjuvant) obtained as described in any of Examples 3 through 6, after which the spleen is returned to the peritoneal cavity and the animal is stitched closed. Three days later, the mouse is sacrificed and its spleen removed. A spleen cell suspension is prepared, washed twice with RPMI 1640 supplemented with 3% fetal calf serum (FCS), and resuspended in 25 ml of the same medium. Myeloma cells (NS-O) are collected at logarithmic growth phase, washed once and added to the spleen cell suspension in a 50 ml tube, at a ratio of 3:1 or 2:1 (spleen cells:myeloma cells). The mixture is pelleted at about 450 g (1500 rpm), the supernatant aspirated, and the pellet loosened by tapping the tube. Fusion is performed at room temperature by adding 1 ml of polyethylene glycol (PEG) 1500 over 1 minute, with constant stirring. The mixture is incubated for another minute, then 1 ml of warm RPMI (30 to 37°C) is added over 1 minute followed by 5 ml RPMI over 3 minutes and another 10 ml RPMI over another 3 minutes. The cell suspension is centrifuged and resuspended in about 200 ml of HAT selective medium consisting of RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 20% FCS, 100 mM hypoxanthine, 0.4 mM aminopterin and 16 mM thymidine. The cell suspension is dispensed in 1 ml volumes into tissue culture plates and incubated at 37°C in a humid atmosphere with 5% CO_2-95% air for 8 to 10 days. Supernatants are aspirated and

the cells are fed with 1 ml HAT medium per well, every 2 to 3 days according to cell growth. Supernatants of confluent wells are screened for specific antibodies and positive wells are cloned.

Example 8

Catalytic Activity of Recombinant Chitinase

Chitotriosidase (chitinase) activity was measured using the fluorogenic substrate 4-methylumbelliferyl-β-D-*N,N',N''*-triacetylchitotriose (4 MU-chitotrioside, Sigma Chemical, St. Louis, MO) in McIlvain buffer (Hollak *et al., supra*). Ten μl samples of the recombinant product were combined with 10 μl bovine serum albumin (10 mg/ml), 15 μl fluorogenic substrate (2.71 mM), and 65 μl buffer (0.1M citric acid, 0.2M sodium phosphate, pH 5.2) in a total volume of 100 μl. Reactions were incubated at 37°C for 15 minutes, then the reaction was stopped with the addition of 2 ml of 0.3M glycine/NaOH buffer (pH 10.6). The fluorescent cleavage product, 4-methylumbelliferone, was monitored with a fluorimeter (SLM-AMINCO Instruments, Inc., Rochester, NY) at 450 nm. To obtain a standard curve, several substrate concentrations were combined with excess bacterial chitinase to ensure that substrate was completely cleaved. The known quantity of 4-MU was then correlated to the fluorescence signal from the fluorimeter and linear regression was used to determine a standard curve. The signal produced with diluted purified recombinant chitinase in the assay was then used to interpolate the nm quantity of substrate cleaved by the enzyme during the reaction time. This number was then divided by the concentration of protein to obtain the nm/min per mg protein (determined by A_{280} and calculated molar extinction coefficient).

The chitotriosidase activity of the recombinant human chitinase from Example 5 was determined to be 90 nm/min per mg protein.

Example 9

Anti-fungal Activity of Recombinant Chitinase *In Vitro*

In a preliminary experiment, recombinant human chitinase was tested for inhibition of fungal growth *in vitro*. The two fungi *Candida albicans* and *Aspergillus fumigatis* are serious pathogens for immunocompromised patients. Both *Candida* and *Aspergillus* were grown in RPMI growth media at 37°C to approximately 10,000-50,000 colony forming units (CFU) per ml. Recombinant human chitinase (prepared as described in Example 5) was added to cultures at 0, 2.8, 11.25, or 45 μg/ml. After 24 hours, fungal growth was assessed by turbidity of cultures. Under these non-physiological conditions in this assay, all cultures appeared to grow at comparable rates, independent of chitinase concentration. The concentration of fungi tested, however, is much higher than the fungal burden seen during fungal infection *in vivo*. Different results may be obtained under different conditions, *e.g.*, with a lower fungal burden, or when human chitinase is tested in combination with other anti-fungal agents. Chitinase is also expected to be more effective *in vivo* under physiological conditions.

Example 10

Anti-fungal Activity of Recombinant Chitinase *In Vivo* in Mice

Several animal models have been developed for testing efficacy of anti-fungal compounds [see Louie et al., *Infect. Immun., 62*: 2761-2772, 1994; Kinsman et al., *Antimicrobial Agents and Chemotherapy, 37*: 1243-1246, 1993; Nakajima et al., *Antimicrobial Agents and Chemotherapy 39*: 1517-1521, 1995; Tonetti et al., *Eur. J. Immunol., 25*:1559-1565 (1995)]. Briefly, the animal host is infected with the fungi, varying doses of chitinase are administered to the animals, and their survival is measured over time. The experiments are performed using chitinase as the sole therapeutic agent, or with a combination of conventional anti-fungal agents such as Amphotericin B and fluconazole to determine if the chitinase improves the efficacy of such compounds. Specifically, acute systemic candidiasis is achieved in mice by intraperitoneal or intravenous challenge of 10 x 10^6 CFU *Candida albicans*. The therapeutic agents are administered before or at 1 to 5 hours after challenge, and the number of survivors is determined after five days. In addition, the mice can be sacrificed and fungal load can be determined in specific organs such as kidney, lung, liver and spleen. Alternatively, the mice are challenged with lower doses of *Candida* (1 x 10^6 CFU), in which case survival can be measured at more distant time points, *e.g.*, 45 days. Effective anti-fungal agents enhance the long term survival of animals and reduce fungal load in blood and organs.

Example 11

Activity of Chitinase *In Vivo* in a Rabbit Model of Invasive Aspergillosis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in an immunosuppressed rabbit model of invasive aspergillosis which has been used for over ten years to evaluate a variety of anti-fungal therapies. See, *e.g.*, Andriole et al., *Clin. Infect. Dis., 14(Suppl. 1):*S134-S138 (1992). The study is conducted generally according to Patterson et al., *Antimicrob. Agents Chemother., 37:*2307-2310 (1993) or George et al., *J. Infect. Dis., 168*:692-698 (1993). Briefly, on day one the rabbits are given cyclophosphamide (200 mg) intravenously to render them leukopenic, followed by triamcinolone acetonide (10 mg) subcutaneously each day for the duration of the experiment. On day two, 24 hours after immunosuppression, the animals are challenged intravenously with about 10^6 (lethal challenge) or about 10^5 (sublethal challenge) *A. fumigatus* conidia. Anti-fungal therapy (chitinase alone, or in combination with other conventional anti-fungal agents, *e.g.*, amphotericin B, fluconazole, or 5-fluorocytosine) is initiated at 24 hours after challenge or 48 hours before challenge (for prophylaxis) and is continued for 5 to 6 days or until death. Exemplary doses of conventional anti-fungal agents are 1.5 or 0.5 mg/kg/day intravenous amphotericin B, 60 or 120 mg/kg/day oral fluconazole and 100 mg/kg/day oral 5-fluorocytosine. Control rabbits are not treated with any anti-fungal agent.

At autopsy or death, semiquantitative fungal cultures and histopathologic examination are conducted on the liver, spleen, kidneys, lungs and brain. Cultures of the heart, urine and blood may also be performed. Blood samples

are obtained at intervals and assayed for white blood cell counts and circulating *Aspergillus* carbohydrate antigen using an ELISA assay. The effect of treatment with the test drug is evaluated on three endpoints: reduction in mortality rate, reduction in number of *Aspergillus* organisms cultured from target organs (fungal burden), and reduction in level of circulating *Aspergillus* antigen. Effective anti-fungal agents reduce mortality and/or fungal load.

Alternatively, pulmonary aspergillosis may be evaluated in this model generally according to Chilvers et al., *Mycopathologia, 108:*163-71 (1989), in which the immunosuppressed rabbits are challenged with intratracheal instillation of *Aspergillus fumigatus* conidia, followed by bronchoalveolar lavage on days 1, 2, 4, 7 and 10 following challenge; fungal culture, chitin assay, white cell counts and histopathology are performed on the lavage fluids to determine infective load within the lung. Effective fungal agents reduce the infective load or inflammation within the lung.

Example 12

Activity of Chitinase *In Vivo* in a Rabbit Model of Disseminated Candidiasis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in a rabbit model of disseminated candidiasis generally according to Rouse et al., *Antimicrob. Agents Chemother., 36*:56-58 (1992). New Zealand white rabbits are infected systemically with about 3 x 10^6 *Candida albicans* blastospores. Anti-fungal therapy is initiated 48 hours after challenge with *Candida* (or before challenge for prophylaxis) and is continued for,

e.g., four days. Surviving animals are sacrificed, and fungal cultures are performed on the aortic valve with attached vegetation, lung, kidney and spleen. Fungal cultures and histopathological examination may also be performed on these and other organs, such as liver, brain, and heart. Urine and blood cultures may also be done. The effect of the anti-fungal therapy on mortality and circulating or tissue fungal burden is determined.

Bayer et al., *Antimicrob. Agents Chemother.*, *19*:179-184 (1981), in which rabbits are inoculated intraperitoneally with about 5 x 10^8 CFU *Candida albicans*. A saline peritoneal aspirate is obtained and cultured from each animal four days after intraperitoneal inoculation, and animals with a positive fungal culture aspirate are randomly assigned to control or treatment groups. Anti-fungal treatment is begun seven days after challenge. The eyes of all rabbits are evaluated using indirect ophthalmoscopy, as disseminated candidiasis may result in *Candida* endophthalmitis. Animals are sacrificed at 7, 11 and 14 days after initiation of therapy and their abdomens inspected for evidence of peritonitis and intraabdominal abscess formation. Eyes are examined for macroscopic lesions. Tissue samples from peritoneal abscesses, all other visible abscesses, kidneys, livers, spleens and ocular structures are weighed, homogenized in brain heart infusion broth, serially diluted and cultured to determine the CFU per gram of tissue. Renal and peritoneal abscesses are also fixed in 10% neutral formaldehyde and examined for histopathology. Sections are stained with periodic acid-Schiff reagent to determine the fungal burden and fungal morphology. Effect of the test drugs on improving survival and reducing fungal burden is evaluated.

Example 13

Activity of Chitinase *In Vivo* in a Rabbit Model of Fungal Endophthalmitis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in a rabbit model of *Candida* endophthalmitis, generally according to Park et al., *Antimicrob. Agents Chemother.*, *39*:958-963 (1995). Briefly, New Zealand albino rabbits, 2 to 2.5 kg, are infected with an intravitreal inoculation of about 1,000 CFU of *Candida albicans*. Endophthalmitis is confirmed 5 days after inoculation by indirect ophthalmoscopy, and is defined as moderate to severe vitreous haze with partial or complete obscuration of greater than 50% of the retinal and choroidal vasculature. The vitreous turbidity is graded on a scale, and the fundus appearance may be graded and documented by fundus photography. The rabbits are then randomized to the following treatment conditions: chitinase alone for 2 to 4 weeks, a combination of chitinase and another conventional anti-fungal agent (*e.g.*, amphotericin B, fluconazole or 5-fluorocytosine) for 2 to 4 weeks, or no treatment (control). Exemplary doses of conventional anti-fungal agents are 80 mg/kg/day of oral fluconazole and 100 mg/kg every 12 hours of oral 5-fluorocytosine.

The treatment effect is assessed at 2 and 4 weeks after therapy by indirect ophthalmoscopy, quantitative fungal culture, and histopathology. For quantitative fungal culture, the eyes are dissected and weighed, and a weighed fraction of each sample is homogenized and cultured on brucella agar-5% horse blood plates for 48 hours at 35°C in 5 to 10% CO_2. The homogenized sample may also be diluted 10- or 100-fold with sterile saline before plating. The colonies are counted

and the total CFU in the eye calculated on the basis of the growth yielded from the measured fractions of sample. Treatment effect is assessed in terms of a reduction in the total intraocular fungal burden. For histopathology, representative eyes are removed, fixed in formalin, embedded in plastic, and sliced into 5 μm sections. The sections are stained with hematoxylin-eosin or Gomori's methenamine silver stain and examined by light microscopy for inflammation, fibrous organization and fungal elements. The effect of the anti-fungal agents on reducing mortality, reducing fungal load, or reducing the inflammation associated with fungal infection, is evaluated.

Alternatively, a rabbit model of *Aspergillus* endophthalmitis may be used generally according to Jain et al., *Doc. Ophthalmol.*,*69*:227-235 (1988). Briefly, New Zealand white rabbits are inoculated in one eye with about forty spores of *Aspergillus fumigatus*. Their contralateral (control) eyes receive a similar but sterile inoculum. After treatment with the test drug (chitinase alone, or chitinase in combination with another agent), the rabbits' eyes may be evaluated for clinical appearance, electroretinogram waveforms, indirect ophthalmoscopy, quantitative fungal culture, and histopathology. Clinically evident endophthalmitis typically develops within three to seven days after inoculation.

Example 14

Activity of Chitinase *In Vivo* in a Rabbit Model of Fungal Endocarditis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in a rabbit model of *Candida* endocarditis generally according to Witt and Bayer, *Antimicrob. Agents Chemother.*, *35*:2481-2485

(1991). See also Longman et al., *Rev. Infect. Dis., 12(Suppl. 3)*:S294-298 (1990). Sterile thrombotic endocarditis is produced in New Zealand white rabbits by transaortic valvular placement of a sterile polyethylene catheter (internal diameter, 0.86 mm), which remained in place for the duration of the study. Infective endocarditis is then established 48 hours after catheterization by intravenous injection of about 2×10^7 *C. albicans* blastospores. Alternatively, *C. parapsilosis* may be used. Anti-fungal therapy (chitinase or chitinase in combination with another conventional anti-fungal agent) is initiated either 24 hours before or 24 to 60 hours after fungal challenge. Therapy is continued daily for 9 or 12 days. Exemplary doses of conventional anti-fungal agents are 1 mg/kg/day intravenous amphotericin B, 50 mg/kg/day or 100 mg/kg/day intravenous or intraperitoneal fluconazole. Control rabbits are given no anti-fungal agent. At sacrifice, hearts are removed and the position of the indwelling catheter verified. Cardiac vegetations from each animal are removed, pooled, weighed and homogenized in 1 ml of sterile saline. The homogenate is serially diluted and quantitatively cultured on yeast potassium dextrose agar at 35°C for 48 hours. Culture-negative vegetations are considered to contain less than 2 $\log_{10}$ CFU/gram on the basis of average vegetation weight.

Numerous modifications and variations of the above-described invention are expected to occur to those of skill in the art. Accordingly, only such limitations as appear in the appended claims should be placed thereon.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Gray, Patrick W.

(ii) TITLE OF INVENTION: Chitinase Materials and Methods

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun
(B) STREET: 6300 Sears Tower, 233 South Wacker Drive
(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: United States of America
(F) ZIP: 60606-6402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 33,547
(C) REFERENCE/DOCKET NUMBER: 27866/32960

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 312/474-6300
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(C) TELEX: 25-3856

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1636 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2..1399

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 65..1399

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Ala Gln Tyr Arg Gln Gly Glu Ala Arg Phe Leu Pro Lys Asp Leu Asp
                15                  20                  25

CCC AGC CTT TGC ACC CAC CTC ATC TAC GCC TTC GCT GGC ATG ACC AAC     190
Pro Ser Leu Cys Thr His Leu Ile Tyr Ala Phe Ala Gly Met Thr Asn
            30                  35                  40

CAC CAG CTG AGC ACC ACT GAG TGG AAT GAC GAG ACT CTC TAC CAG GAG     238
His Gln Leu Ser Thr Thr Glu Trp Asn Asp Glu Thr Leu Tyr Gln Glu
        45                  50                  55

TTC AAT GGC CTG AAG AAG ATG AAT CCC AAG CTG AAG ACC CTG TTA GCC     286
Phe Asn Gly Leu Lys Lys Met Asn Pro Lys Leu Lys Thr Leu Leu Ala
    60                  65                  70

ATC GGA GGC TGG AAT TTC GGC ACT CAG AAG TTC ACA GAT ATG GTA GCC     334
Ile Gly Gly Trp Asn Phe Gly Thr Gln Lys Phe Thr Asp Met Val Ala
75                  80                  85                  90

ACG GCC AAC AAC CGT CAG ACC TTT GTC AAC TCG GCC ATC AGG TTT CTG     382
Thr Ala Asn Asn Arg Gln Thr Phe Val Asn Ser Ala Ile Arg Phe Leu
                95                  100                 105

CGC AAA TAC AGC TTT GAC GGC CTT GAC CTT GAC TGG GAG TAC CCA GGA     430
Arg Lys Tyr Ser Phe Asp Gly Leu Asp Leu Asp Trp Glu Tyr Pro Gly
            110                 115                 120

AGC CAG GGG AGC CCT GCC GTA GAC AAG GAG CGC TTC ACA ACC CTG GTA     478
Ser Gln Gly Ser Pro Ala Val Asp Lys Glu Arg Phe Thr Thr Leu Val
        125                 130                 135

CAG GAC TTG GCC AAT GCC TTC CAG CAG GAA GCC CAG ACC TCA GGG AAG     526
Gln Asp Leu Ala Asn Ala Phe Gln Gln Glu Ala Gln Thr Ser Gly Lys
    140                 145                 150

GAA CGC CTT CTT CTG AGT GCA GCG GTT CCA GCT GGG CAG ACC TAT GTG     574
Glu Arg Leu Leu Leu Ser Ala Ala Val Pro Ala Gly Gln Thr Tyr Val
155                 160                 165                 170

GAT GCT GGA TAC GAG GTG GAC AAA ATC GCC CAG AAC CTG GAT TTT GTC     622
Asp Ala Gly Tyr Glu Val Asp Lys Ile Ala Gln Asn Leu Asp Phe Val
                175                 180                 185

AAC CTT ATG GCC TAC GAC TTC CAT GGC TCT TGG GAG AAG GTC ACG GGA     670
Asn Leu Met Ala Tyr Asp Phe His Gly Ser Trp Glu Lys Val Thr Gly
            190                 195                 200

CAT AAC AGC CCC CTC TAC AAG AGG CAA GAA GAG AGT GGT GCA GCA GCC     718
His Asn Ser Pro Leu Tyr Lys Arg Gln Glu Glu Ser Gly Ala Ala Ala
        205                 210                 215

AGC CTC AAC GTG GAT GCT GCT GTG CAA CAG TGG CTG CAG AAG GGG ACC     766
Ser Leu Asn Val Asp Ala Ala Val Gln Gln Trp Leu Gln Lys Gly Thr
    220                 225                 230

CCT GCC AGC AAG CTG ATC CTT GGC ATG CCT ACC TAC GGA CGC TCC TTC     814
Pro Ala Ser Lys Leu Ile Leu Gly Met Pro Thr Tyr Gly Arg Ser Phe
235                 240                 245                 250

ACA CTG GCC TCC TCA TCA GAC ACC AGA GTG GGG GCC CCA GCC ACA GGG     862
Thr Leu Ala Ser Ser Ser Asp Thr Arg Val Gly Ala Pro Ala Thr Gly
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                    255                 260                 265

TCT GGC ACT CCA GGC CCC TTC ACC AAG GAA GGA GGG ATG CTG GCC TAC      910
Ser Gly Thr Pro Gly Pro Phe Thr Lys Glu Gly Gly Met Leu Ala Tyr
            270                 275                 280

TAT GAA GTC TGC TCC TGG AAG GGG GCC ACC AAA CAG AGA ATC CAG GAT      958
Tyr Glu Val Cys Ser Trp Lys Gly Ala Thr Lys Gln Arg Ile Gln Asp
        285                 290                 295

CAG AAG GTG CCC TAC ATC TTC CGG GAC AAC CAG TGG GTG GGC TTT GAT     1006
Gln Lys Val Pro Tyr Ile Phe Arg Asp Asn Gln Trp Val Gly Phe Asp
    300                 305                 310

GAT GTG GAG AGC TTC AAA ACC AAG GTC AGC TAT CTG AAG CAG AAG GGA     1054
Asp Val Glu Ser Phe Lys Thr Lys Val Ser Tyr Leu Lys Gln Lys Gly
315                 320                 325                 330

CTG GGC GGG GCC ATG GTC TGG GCA CTG GAC TTA GAT GAC TTT GCC GGC     1102
Leu Gly Gly Ala Met Val Trp Ala Leu Asp Leu Asp Asp Phe Ala Gly
                335                 340                 345

TTC TCC TGC AAC CAG GGC CGA TAC CCC CTC ATC CAG ACG CTA CGG CAG     1150
Phe Ser Cys Asn Gln Gly Arg Tyr Pro Leu Ile Gln Thr Leu Arg Gln
            350                 355                 360

GAA CTG AGT CTT CCA TAC TTG CCT TCA GGC ACC CCA GAG CTT GAA GTT     1198
Glu Leu Ser Leu Pro Tyr Leu Pro Ser Gly Thr Pro Glu Leu Glu Val
        365                 370                 375

CCA AAA CCA GGT CAG CCC TCT GAA CCT GAG CAT GGC CCC AGC CCT GGA     1246
Pro Lys Pro Gly Gln Pro Ser Glu Pro Glu His Gly Pro Ser Pro Gly
    380                 385                 390

CAA GAC ACG TTC TGC CAG GGC AAA GCT GAT GGG CTC TAT CCC AAT CCT     1294
Gln Asp Thr Phe Cys Gln Gly Lys Ala Asp Gly Leu Tyr Pro Asn Pro
395                 400                 405                 410

CGG GAA CGG TCC AGC TTC TAC AGC TGT GCA GCG GGG CGG CTG TTC CAG     1342
Arg Glu Arg Ser Ser Phe Tyr Ser Cys Ala Ala Gly Arg Leu Phe Gln
                415                 420                 425

CAA AGC TGC CCG ACA GGC CTG GTG TTC AGC AAC TCC TGC AAA TGC TGC     1390
Gln Ser Cys Pro Thr Gly Leu Val Phe Ser Asn Ser Cys Lys Cys Cys
            430                 435                 440

ACC TGG AAT TGAGTCGCTA AAGCCCCTCC AGTCCCAGCT TTGAGGCTGG             1439
Thr Trp Asn
        445

GCCCAGGATC ACTCTACAGC CTGCCTCCTG GGTTTTCCCT GGGGGCCGCA ATCTGGCTCC   1499

TGCAGGCCTT TCTGTGGTCT TCCTTTATCC AGGCTTTCTG CTCTCAGCCT TGCCTTCCTT   1559

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 466 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Val Arg Ser Val Ala Trp Ala Gly Phe Met Val Leu Leu Met Ile
-21 -20 -15 -10

Pro Trp Gly Ser Ala Ala Lys Leu Val Cys Tyr Phe Thr Asn Trp Ala
-5 1 5 10

Gln Tyr Arg Gln Gly Glu Ala Arg Phe Leu Pro Lys Asp Leu Asp Pro
15 20 25

Ser Leu Cys Thr His Leu Ile Tyr Ala Phe Ala Gly Met Thr Asn His
30 35 40

Gln Leu Ser Thr Thr Glu Trp Asn Asp Glu Thr Leu Tyr Gln Glu Phe
45 50 55

Asn Gly Leu Lys Lys Met Asn Pro Lys Leu Lys Thr Leu Leu Ala Ile
60 65 70 75

Gly Gly Trp Asn Phe Gly Thr Gln Lys Phe Thr Asp Met Val Ala Thr
80 85 90

Ala Asn Asn Arg Gln Thr Phe Val Asn Ser Ala Ile Arg Phe Leu Arg
95 100 105

Lys Tyr Ser Phe Asp Gly Leu Asp Leu Asp Trp Glu Tyr Pro Gly Ser
110 115 120

Gln Gly Ser Pro Ala Val Asp Lys Glu Arg Phe Thr Thr Leu Val Gln
125 130 135

Asp Leu Ala Asn Ala Phe Gln Gln Glu Ala Gln Thr Ser Gly Lys Glu
140 145 150 155

Arg Leu Leu Leu Ser Ala Ala Val Pro Ala Gly Gln Thr Tyr Val Asp
160 165 170

Ala Gly Tyr Glu Val Asp Lys Ile Ala Gln Asn Leu Asp Phe Val Asn
175 180 185

Leu Met Ala Tyr Asp Phe His Gly Ser Trp Glu Lys Val Thr Gly His
190 195 200

Asn Ser Pro Leu Tyr Lys Arg Gln Glu Glu Ser Gly Ala Ala Ala Ser
205 210 215

Leu Asn Val Asp Ala Ala Val Gln Gln Trp Leu Gln Lys Gly Thr Pro
220 225 230 235

Ala Ser Lys Leu Ile Leu Gly Met Pro Thr Tyr Gly Arg Ser Phe Thr
240 245 250

Leu Ala Ser Ser Ser Asp Thr Arg Val Gly Ala Pro Ala Thr Gly Ser
255 260 265

Gly Thr Pro Gly Pro Phe Thr Lys Glu Gly Gly Met Leu Ala Tyr Tyr
270 275 280

Glu Val Cys Ser Trp Lys Gly Ala Thr Lys Gln Arg Ile Gln Asp Gln
285 290 295

Lys Val Pro Tyr Ile Phe Arg Asp Asn Gln Trp Val Gly Phe Asp Asp

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300                 305                 310                 315

Val Glu Ser Phe Lys Thr Lys Val Ser Tyr Leu Lys Gln Lys Gly Leu
                320                 325                 330

Gly Gly Ala Met Val Trp Ala Leu Asp Leu Asp Asp Phe Ala Gly Phe
            335                 340                 345

Ser Cys Asn Gln Gly Arg Tyr Pro Leu Ile Gln Thr Leu Arg Gln Glu
        350                 355                 360

Leu Ser Leu Pro Tyr Leu Pro Ser Gly Thr Pro Glu Leu Glu Val Pro
    365                 370                 375

Lys Pro Gly Gln Pro Ser Glu Pro Glu His Gly Pro Ser Pro Gly Gln
380                 385                 390                 395

Asp Thr Phe Cys Gln Gly Lys Ala Asp Gly Leu Tyr Pro Asn Pro Arg
                400                 405                 410

Glu Arg Ser Ser Phe Tyr Ser Cys Ala Ala Gly Arg Leu Phe Gln Gln
            415                 420                 425

Ser Cys Pro Thr Gly Leu Val Phe Ser Asn Ser Cys Lys Cys Cys Thr
        430                 435                 440

Trp Asn
    445
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1656 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 27..1424

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 90..1424

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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TGC TAC TTC ACC AAC TGG GCC CAG TAC AGA CAG GGG GAG GCT CGC TTC       149
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  5                  10                  15                  20

CTG CCC AAG GAC TTG GAC CCC AGC CTT TGC ACC CAC CTC ATC TAC GCC       197
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                25                  30                  35
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TTC GCT GGC ATG ACC AAC CAC CAG CTG AGC ACC ACT GAG TGG AAT GAC          245
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GAG ACT CTC TAC CAG GAG TTC AAT GGC CTG AAG AAG ATG AAT CCC AAG          293
Glu Thr Leu Tyr Gln Glu Phe Asn Gly Leu Lys Lys Met Asn Pro Lys
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CTG AAG ACC CTG TTA GCC ATC GGA GGC TGG AAT TTC AGC ACT CAG AAG          341
Leu Lys Thr Leu Leu Ala Ile Gly Gly Trp Asn Phe Ser Thr Gln Lys
     70                  75                  80

TTC ACA GAT ATG GTA GCC ACG GCC AAC AAC CGT CAG ACC TTT GTC AAC          389
Phe Thr Asp Met Val Ala Thr Ala Asn Asn Arg Gln Thr Phe Val Asn
 85                  90                  95                 100

TCG GCC ATC AGG TTT CTG CGC AAA TAC AGC TTT GAC GGC CTT GAC CTT          437
Ser Ala Ile Arg Phe Leu Arg Lys Tyr Ser Phe Asp Gly Leu Asp Leu
                105                 110                 115

GAC TGG GAG TAC CCA GGA AGC CAG GGG AGC CCT GCC GTA GAC AAG GAG          485
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          120                 125                 130

CGC TTC ACA ACC CTG GTA CAG GAC TTG GCC AAT GCC TTC CAG CAG GAA          533
Arg Phe Thr Thr Leu Val Gln Asp Leu Ala Asn Ala Phe Gln Gln Glu
     135                 140                 145

GCC CAG ACC TCA GGG AAG GAA CGC CTT CTT CTG AGT GCA GCG GTT CCA          581
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GCT GGG CAG ACC TAT GTG GAT GCT GGA TAC GAG GTG GAC AAA ATC GCC          629
Ala Gly Gln Thr Tyr Val Asp Ala Gly Tyr Glu Val Asp Lys Ile Ala
165                 170                 175                 180

CAG AAC CTG GAT TTT GTC AAC CTT ATG GCC TAC GAC TTC CAT GGC TCT          677
Gln Asn Leu Asp Phe Val Asn Leu Met Ala Tyr Asp Phe His Gly Ser
                185                 190                 195

TGG GAG AAG GTC ACG GGA CAT AAC AGC CCC CTC TAC AAG AGG CAA GAA          725
Trp Glu Lys Val Thr Gly His Asn Ser Pro Leu Tyr Lys Arg Gln Glu
          200                 205                 210

GAG AGT GGT GCA GCA GCC AGC CTC AAC GTG GAT GCT GCT GTG CAA CAG          773
Glu Ser Gly Ala Ala Ala Ser Leu Asn Val Asp Ala Ala Val Gln Gln
     215                 220                 225

TGG CTG CAG AAG GGG ACC CCT GCC AGC AAG CTG ATC CTT GGC ATG CCT          821
Trp Leu Gln Lys Gly Thr Pro Ala Ser Lys Leu Ile Leu Gly Met Pro
230                 235                 240

ACC TAC GGA CGC TCC TTC ACA CTG GCC TCC TCA TCA GAC ACC AGA GTG          869
Thr Tyr Gly Arg Ser Phe Thr Leu Ala Ser Ser Ser Asp Thr Arg Val
245                 250                 255                 260

GGG GCC CCA GCC ACA GGG TCT GGC ACT CCA GGC CCC TTC ACC AAG GAA          917
Gly Ala Pro Ala Thr Gly Ser Gly Thr Pro Gly Pro Phe Thr Lys Glu
                265                 270                 275

GGA GGG ATG CTG GCC TAC TAT GAA GTC TGC TCC TGG AAG GGG GCC ACC          965
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          280                 285                 290

AAA CAG AGA ATC CAG GAT CAG AAG GTG CCC TAC ATC TTC CGG GAC AAC         1013
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Lys Gln Arg Ile Gln Asp Gln Lys Val Pro Tyr Ile Phe Arg Asp Asn
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Gln Trp Val Gly Phe Asp Asp Val Glu Ser Phe Lys Thr Lys Val Ser
    310                 315                 320

TAT CTG AAG CAG AAG GGA CTG GGC GGG GCC ATG GTC TGG GCA CTG GAC      1109
Tyr Leu Lys Gln Lys Gly Leu Gly Gly Ala Met Val Trp Ala Leu Asp
325                 330                 335                 340

TTA GAT GAC TTT GCC GGC TTC TCC TGC AAC CAG GGC CGA TAC CCC CTC      1157
Leu Asp Asp Phe Ala Gly Phe Ser Cys Asn Gln Gly Arg Tyr Pro Leu
                345                 350                 355

ATC CAG ACG CTA CGG CAG GAA CTG AGT CTT CCA TAC TTG CCT TCA GGC      1205
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            360                 365                 370

ACC CCA GAG CTT GAA GTT CCA AAA CCA GGT CAG CCC TCT GAA CCT GAG      1253
Thr Pro Glu Leu Glu Val Pro Lys Pro Gly Gln Pro Ser Glu Pro Glu
        375                 380                 385

CAT GGC CCC AGC CCT GGA CAA GAC ACG TTC TGC CAG GGC AAA GCT GAT      1301
His Gly Pro Ser Pro Gly Gln Asp Thr Phe Cys Gln Gly Lys Ala Asp
    390                 395                 400

GGG CTC TAT CCC AAT CCT CGG GAA CGG TCC AGC TTC TAC AGC TGT GCA      1349
Gly Leu Tyr Pro Asn Pro Arg Glu Arg Ser Ser Phe Tyr Ser Cys Ala
405                 410                 415                 420

GCG GGG CGG CTG TTC CAG CAA AGC TGC CCG ACA GGC CTG GTG TTC AGC      1397
Ala Gly Arg Leu Phe Gln Gln Ser Cys Pro Thr Gly Leu Val Phe Ser
                425                 430                 435

AAC TCC TGC AAA TGC TGC ACC TGG AAT TGAGTCGCTA AAGCCCCTCC             1444
Asn Ser Cys Lys Cys Cys Thr Trp Asn
            440                 445

AGTCCCAGCT TTGAGGCTGG GCCCAGGATC ACTCTACAGC CTGCCTCCTG GGTTTTCCCT    1504

GGGGGCCGCA ATCTGGCTCC TGCAGGCCTT TCTGTGGTCT TCCTTTATCC AGGCTTTCTG    1564

CTCTCAGCCT TGCCTTCCTT TTTTCTGGGT CTCCTGGGCT GCCCCTTTCA CTTGCAAAAT    1624

AAATCTTTGG TTTGTGCCCC TCAAAAAAAA AA                                   1656
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 466 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Gln Tyr Arg Gln Gly Glu Ala Arg Phe Leu Pro Lys Asp Leu Asp Pro
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                15                  20                  25
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          30                  35                  40
Gln Leu Ser Thr Thr Glu Trp Asn Asp Glu Thr Leu Tyr Gln Glu Phe
     45                  50                  55
Asn Gly Leu Lys Lys Met Asn Pro Lys Leu Lys Thr Leu Leu Ala Ile
 60                  65                  70                  75
Gly Gly Trp Asn Phe Ser Thr Gln Lys Phe Thr Asp Met Val Ala Thr
                 80                  85                  90
Ala Asn Asn Arg Gln Thr Phe Val Asn Ser Ala Ile Arg Phe Leu Arg
            95                  100                 105
Lys Tyr Ser Phe Asp Gly Leu Asp Leu Asp Trp Glu Tyr Pro Gly Ser
        110                 115                 120
Gln Gly Ser Pro Ala Val Asp Lys Glu Arg Phe Thr Thr Leu Val Gln
    125                 130                 135
Asp Leu Ala Asn Ala Phe Gln Gln Glu Ala Gln Thr Ser Gly Lys Glu
140                 145                 150                 155
Arg Leu Leu Leu Ser Ala Ala Val Pro Ala Gly Gln Thr Tyr Val Asp
                160                 165                 170
Ala Gly Tyr Glu Val Asp Lys Ile Ala Gln Asn Leu Asp Phe Val Asn
            175                 180                 185
Leu Met Ala Tyr Asp Phe His Gly Ser Trp Glu Lys Val Thr Gly His
        190                 195                 200
Asn Ser Pro Leu Tyr Lys Arg Gln Glu Glu Ser Gly Ala Ala Ala Ser
    205                 210                 215
Leu Asn Val Asp Ala Ala Val Gln Gln Trp Leu Gln Lys Gly Thr Pro
220                 225                 230                 235
Ala Ser Lys Leu Ile Leu Gly Met Pro Thr Tyr Gly Arg Ser Phe Thr
                240                 245                 250
Leu Ala Ser Ser Ser Asp Thr Arg Val Gly Ala Pro Ala Thr Gly Ser
            255                 260                 265
Gly Thr Pro Gly Pro Phe Thr Lys Glu Gly Gly Met Leu Ala Tyr Tyr
        270                 275                 280
Glu Val Cys Ser Trp Lys Gly Ala Thr Lys Gln Arg Ile Gln Asp Gln
    285                 290                 295
Lys Val Pro Tyr Ile Phe Arg Asp Asn Gln Trp Val Gly Phe Asp Asp
300                 305                 310                 315
Val Glu Ser Phe Lys Thr Lys Val Ser Tyr Leu Lys Gln Lys Gly Leu
                320                 325                 330
Gly Gly Ala Met Val Trp Ala Leu Asp Leu Asp Asp Phe Ala Gly Phe
            335                 340                 345
Ser Cys Asn Gln Gly Arg Tyr Pro Leu Ile Gln Thr Leu Arg Gln Glu
        350                 355                 360
```

Leu Ser Leu Pro Tyr Leu Pro Ser Gly Thr Pro Glu Leu Glu Val Pro
365 370 375

Lys Pro Gly Gln Pro Ser Glu Pro Glu His Gly Pro Ser Pro Gly Gln
380 385 390 395

Asp Thr Phe Cys Gln Gly Lys Ala Asp Gly Leu Tyr Pro Asn Pro Arg
400 405 410

Glu Arg Ser Ser Phe Tyr Ser Cys Ala Ala Gly Arg Leu Phe Gln Gln
415 420 425

Ser Cys Pro Thr Gly Leu Val Phe Ser Asn Ser Cys Lys Cys Cys Thr
430 435 440

Trp Asn
445

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACACTATAG AATAGGGC 18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGGGATCATC AGCAGGACCA TGAAACCTGC CCAGGCCACA GACCGCACCA T 51

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TACATCTAGA ATTATGGCAA AACTGGTCTG CTACTTCACC 40

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGATCTAACC TTAGGTGCCT GAAGACAAGT ATGG 34

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TACAGAATTC TTATTCACAT CCGGCCCTG 29

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TACATCTAGA CTCCATCCAG AAAAACAGGT ATGG 34

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCTAGAGTCG ACCTGCAGGC ATGCAAGCTT 30

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCAAGCTTG AGAGCTCCGT TCCGCCACAT GGTGCGGTCT GTGGCCTGGG 50

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GACTCTAGAC TAGGTGCCTG AAGGCAAGTA TG 32

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 373 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Ala Lys Leu Val Cys Tyr Phe Thr Asn Trp Ala Gln Tyr Arg Gln Gly
1               5                   10                  15

Glu Ala Arg Phe Leu Pro Lys Asp Leu Asp Pro Ser Leu Cys Thr His
            20                  25                  30

Leu Ile Tyr Ala Phe Ala Gly Met Thr Asn His Gln Leu Ser Thr Thr
        35                  40                  45

Glu Trp Asn Asp Glu Thr Leu Tyr Gln Glu Phe Asn Gly Leu Lys Lys
    50                  55                  60

Met Asn Pro Lys Leu Lys Thr Leu Leu Ala Ile Gly Gly Trp Asn Phe
65                  70                  75                  80

Gly Thr Gln Lys Phe Thr Asp Met Val Ala Thr Ala Asn Asn Arg Gln
                85                  90                  95

Thr Phe Val Asn Ser Ala Ile Arg Phe Leu Arg Lys Tyr Ser Phe Asp
            100                 105                 110

Gly Leu Asp Leu Asp Trp Glu Tyr Pro Gly Ser Gln Gly Ser Pro Ala
        115                 120                 125

Val Asp Lys Glu Arg Phe Thr Thr Leu Val Gln Asp Leu Ala Asn Ala
    130                 135                 140

Phe Gln Gln Glu Ala Gln Thr Ser Gly Lys Glu Arg Leu Leu Leu Ser
145                 150                 155                 160

Ala Ala Val Pro Ala Gly Gln Thr Tyr Val Asp Ala Gly Tyr Glu Val
                165                 170                 175

Asp Lys Ile Ala Gln Asn Leu Asp Phe Val Asn Leu Met Ala Tyr Asp
            180                 185                 190

Phe His Gly Ser Trp Glu Lys Val Thr Gly His Asn Ser Pro Leu Tyr
        195                 200                 205
```

Lys Arg Gln Glu Glu Ser Gly Ala Ala Ala Ser Leu Asn Val Asp Ala
210 215 220

Ala Val Gln Gln Trp Leu Gln Lys Gly Thr Pro Ala Ser Lys Leu Ile
225 230 235 240

Leu Gly Met Pro Thr Tyr Gly Arg Ser Phe Thr Leu Ala Ser Ser Ser
245 250 255

Asp Thr Arg Val Gly Ala Pro Ala Thr Gly Ser Gly Thr Pro Gly Pro
260 265 270

Phe Thr Lys Glu Gly Gly Met Leu Ala Tyr Tyr Glu Val Cys Ser Trp
275 280 285

Lys Gly Ala Thr Lys Gln Arg Ile Gln Asp Gln Lys Val Pro Tyr Ile
290 295 300

Phe Arg Asp Asn Gln Trp Val Gly Phe Asp Asp Val Glu Ser Phe Lys
305 310 315 320

Thr Lys Val Ser Tyr Leu Lys Gln Lys Gly Leu Gly Gly Ala Met Val
325 330 335

Trp Ala Leu Asp Leu Asp Asp Phe Ala Gly Phe Ser Cys Asn Gln Gly
340 345 350

Arg Tyr Pro Leu Ile Gln Thr Leu Arg Gln Glu Leu Ser Leu Pro Tyr
355 360 365

Leu Pro Ser Gly Thr
370

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 373 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ala Lys Leu Val Cys Tyr Phe Thr Asn Trp Ala Gln Tyr Arg Gln Gly
1 5 10 15

Glu Ala Arg Phe Leu Pro Lys Asp Leu Asp Pro Ser Leu Cys Thr His
20 25 30

Leu Ile Tyr Ala Phe Ala Gly Met Thr Asn His Gln Leu Ser Thr Thr
35 40 45

Glu Trp Asn Asp Glu Thr Leu Tyr Gln Glu Phe Asn Gly Leu Lys Lys
50 55 60

Met Asn Pro Lys Leu Lys Thr Leu Leu Ala Ile Gly Gly Trp Asn Phe
65 70 75 80

Gly Thr Gln Lys Phe Thr Asp Met Val Ala Thr Ala Asn Asn Arg Gln
85 90 95

Thr Phe Val Asn Ser Ala Ile Arg Phe Leu Arg Lys Tyr Ser Phe Asp
100 105 110

```
Gly Leu Asp Leu Asp Trp Glu Tyr Pro Gly Ser Gln Gly Ser Pro Ala
        115                 120                 125

Val Asp Lys Glu Arg Phe Thr Thr Leu Val Gln Asp Leu Ala Asn Ala
    130                 135                 140

Phe Gln Gln Glu Ala Gln Thr Ser Gly Lys Glu Arg Leu Leu Leu Ser
145                 150                 155                 160

Ala Ala Val Pro Ala Gly Gln Thr Tyr Val Asp Ala Gly Tyr Glu Val
                165                 170                 175

Asp Lys Ile Ala Gln Asn Leu Asp Phe Val Asn Leu Met Ala Tyr Asp
            180                 185                 190

Phe His Gly Ser Trp Glu Lys Val Thr Gly His Asn Ser Pro Leu Tyr
        195                 200                 205

Lys Arg Gln Glu Glu Ser Gly Ala Ala Ala Ser Leu Asn Val Asp Ala
    210                 215                 220

Ala Val Gln Gln Trp Leu Gln Lys Gly Thr Pro Ala Ser Lys Leu Ile
225                 230                 235                 240

Leu Gly Met Pro Thr Tyr Gly Arg Ser Phe Thr Leu Ala Ser Ser Ser
                245                 250                 255

Asp Thr Arg Val Gly Ala Pro Ala Thr Gly Ser Gly Thr Pro Gly Pro
            260                 265                 270

Phe Thr Lys Glu Gly Gly Met Leu Ala Tyr Tyr Glu Val Cys Ser Trp
        275                 280                 285

Lys Gly Ala Thr Lys Gln Arg Ile Gln Asp Gln Lys Val Pro Tyr Ile
    290                 295                 300

Phe Arg Asp Asn Gln Trp Val Gly Phe Asp Asp Val Glu Ser Phe Lys
305                 310                 315                 320

Thr Lys Val Ser Tyr Leu Lys Gln Lys Gly Leu Gly Gly Ala Met Val
                325                 330                 335

Trp Ala Leu Asp Leu Asp Asp Phe Ala Gly Phe Ser Cys Asn Gln Gly
            340                 345                 350

Arg Tyr Pro Leu Ile Gln Thr Leu Arg Gln Glu Leu Ser Leu Pro Tyr
        355                 360                 365

Leu Ser Ser Gly Thr
    370
```

CLAIMS

What is claimed is:

1. A purified, isolated polynucleotide encoding the human chitinase amino acid sequence of SEQ ID NO: 2.

2. The polynucleotide of claim 1 which is a DNA.

3. The DNA of claim 2 comprising the protein coding nucleotides of SEQ ID NO: 1.

4. A purified, isolated polynucleotide encoding amino acids 1 to 445 of SEQ ID NO: 2.

5. The polynucleotide of claim 4 which is a DNA.

6. The DNA of claim 5 comprising nucleotides 65 to 1402 of SEQ ID NO: 1.

7. A purified, isolated polynucleotide encoding the human chitinase amino acid sequence of SEQ ID NO: 4.

8. The polynucleotide of claim 7 which is a DNA.

9. The DNA of claim 8 comprising the protein coding nucleotides of SEQ ID NO: 3.

10. A purified, isolated polynucleotide encoding amino acids 1 to 445 of SEQ ID NO: 4.

11. The polynucleotide of claim 10 which is a DNA.

12. The DNA of claim 11 comprising nucleotides 90 to 1427 of SEQ ID NO: 3.

13. A purified, isolated polynucleotide encoding human chitinase selected from the group consisting of:

(a) a double-stranded DNA comprising the protein coding portions of the sequence set out in SEQ ID NO: 1;

(b) a DNA which hybridizes under stringent conditions to a non-coding strand of the DNA of (a); and

(c) a DNA which, but for the redundancy of the genetic code, would hybridize under stringent conditions to a non-coding strand of DNA sequence of (a) or (b).

14. The polynucleotide of claim 13 which is a DNA.

15. A vector comprising the DNA of claim 2, 3, 5, 6, 8, 9, 11, 12, or 14.

16. The vector of claim 15 that is an expression vector, wherein the DNA is operatively linked to an expression control DNA sequence.

17. A host cell stably transformed or transfected with the DNA of claim 2, 3, 5, 6, 8, 9, 11, 12, or 14 in a manner allowing the expression in said host cell of human chitinase.

18. A method for producing human chitinase comprising culturing the host cell of claim 17 in a nutrient medium and isolating human chitinase from said host cell or said nutrient medium.

19. A purified, isolated polypeptide produced by the method of claim 18.

20. A purified, isolated polypeptide comprising the human chitinase amino acid sequence of SEQ ID NO: 2.

21. A purified, isolated polypeptide comprising the human chitinase amino acid sequence of SEQ ID NO: 4.

22. A purified, isolated polypeptide comprising human chitinase amino acids 1 to 445 of SEQ ID NO: 2.

23. A purified, isolated polypeptide comprising human chitinase amino acids 1 to 445 of SEQ ID NO: 4.

24. A human chitinase fragment lacking from 1 to about 72 C-terminal amino acid residues of mature human chitinase.

25. The human chitinase fragment of SEQ ID NO: 14.

26. A purified, isolated polynucleotide comprising a polynucleotide sequence encoding the amino acid sequence of SEQ ID NO: 14.

27. The polynucleotide of claim 26 which is a DNA.

28. The human chitinase analog of SEQ ID NO: 15.

29. A purified, isolated polynucleotide encoding the amino acid sequence of SEQ ID NO: 15.

30. A hybridoma cell line producing a monoclonal antibody that is specifically reactive with the polypeptide of claims 19, 20, 21, 22, 23 or 28.

31. The monoclonal antibody produced by the hybridoma of claim 30.

ABSTRACT

The present invention provides purified and isolated polynucleotide sequences encoding human chitinase. Also provided are materials and methods for the recombinant production of human chitinase products which are expected to be useful as products for treating fungal infections or for development of products useful for treating the same.

Atty. Docket No: 27866/32960

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled "**CHITINASE MATERIALS AND METHODS**," the specification of which (check one): ☐ is attached hereto; ☒ was filed on June 14, 1996 as Application Serial No. 08/663,618 and was amended on _____ (if applicable); ☐ was filed as PCT International Application No. _____ on _____ and was amended under Article 19 on _____ (if applicable). I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

(Application Serial Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed ☐ Yes ☐ No
(Application Serial Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below:

(Application Serial Number)	(Day/Month/Year Filed)
(Application Serial Number)	(Day/Month/Year Filed)

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

(Application Serial Number)	(Day/Month/Year Filed)	(Status-Patented, Pending or Abandoned)
(Application Serial Number)	(Day/Month/Year Filed)	(Status-Patented, Pending or Abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I hereby appoint as my attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

Alvin D. Shulman (19,412)
Donald J. Brott (19,490)
Owen J. Murray (22,111)
Allen H. Gerstein (22,218)
Nate F. Scarpelli (22,320)
Edward M. O'Toole (22,477)
Michael F. Borun (25,447)
Trevor B. Joike (25,542)
Timothy J. Vezeau (26,348)
Carl E. Moore, Jr. (26,487)
Richard H. Anderson (26,526)
Patrick D. Ertel (26,877)
James P. Zeller (28,491)
William E. McCracken (30,195)
Richard A. Schnurr (30,890)
Anthony Nimmo (30,920)
Christine A. Dudzik (31,245)
Kevin D. Hogg (31,839)
Jeffrey S. Sharp (31,879)
Donald J. Pochopien (32,167)
Martin J. Hirsch (32,237)
James J. Napoli (32,361)
Richard M. La Barge (32,254)
Jeffry W. Smith (33,455)
Douglass C. Hochstetler (33,710)
Cynthia L. Schaller (34,245)
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Send correspondence to: Li-Hsien Rin-Laures

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Full Name of First or Sole Inventor	Citizenship
Patrick W. Gray	United States
Residence Address - Street	**Post Office Address - Street**
2244 38th Place East	2244 38th Place East
City (Zip)	**City (Zip)**
Seattle, 98112 WA	Seattle, 98112
State or Country	**State or Country**
Washington	Washington
Date ☒ August 19, 1996	**Signature** ☒ Patrick W. Gray

☐ See second page for additional inventor(s)

See reverse for relevant rules & statutes

PATENT

Attorney's Docket No: 27866/32960

Applicant or Patentee:	Patrick W. Gray
Serial or Patent No:	08/663,618
Filed or Issued:	June 14, 1996
For:	Chitinase Materials and Methods

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c)) -- SMALL BUSINESS CONCERN

I hereby declare that I am

The owner of the small business concern identified below:

☒ An official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN ICOS Corporation

ADDRESS OF BUSINESS 22021 20th Avenue, S.E.,
Bothell, Washington 98021

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to, and remain with, the small business concern identified above with regard to the invention, entitled Chitinase Materials and Methods, by inventor(s) Patrick W. Gray,

~~SE18095 11/14/96 08663618~~

described in

The specification filed herewith.

☒ Application Serial No. 08/663,618, filed June 14, 1996.

Patent No. ___________, issued _______________.

If the rights held by the above-identified small business concern are not exclusive,

*NOTE: *Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).*

NAME: _____

ADDRESS: _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME: _____

ADDRESS: _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Judith Woods

TITLE OF PERSON OTHER THAN OWNER: Patent and Licensing Counsel

ADDRESS OF PERSON SIGNING: 22021 20th Avenue, S.E.,
Bothell, Washington, 98021

SIGNATURE: Judith A. Woods Date 8-13-96

7. **Deposit Account and Refund Authorization**

The Commissioner is hereby authorized to charge any deficiency in the amount enclosed or any additional fees which may be required during the pendency of this application under 37 CFR 1.16 or 37 CFR 1.17 or under other applicable rules (except payment of issue fees), to Deposit Account No. 13-2855. A copy of this Transmittal is enclosed.

Please refund any overpayment to Marshall, O'Toole, Gerstein, Murray & Borun at the address below.

Please direct all future communications to Li-Hsien Rin-Laures, at the address below.

Respectfully submitted,

MARSHALL, O'TOOLE, GERSTEIN,
MURRAY & BORUN
6300 Sears Tower
233 South Wacker Drive
Chicago, Illinois 60606-6402
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By: Li-Hsien Rin-Laures

Li-Hsien Rin-Laures
Reg. No: 33,547

June 14, 1996

PATENT

Attorney's Docket No: 27866/32960

Applicant or Patentee:	Patrick W. Gray
Serial or Patent No:	08/663,618
Filed or Issued:	June 14, 1996
For:	Chitinase Materials and Methods

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c)) -- SMALL BUSINESS CONCERN

I hereby declare that I am

The owner of the small business concern identified below:

☒ An official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN ICOS Corporation

ADDRESS OF BUSINESS 22021 20th Avenue, S.E.,
Bothell, Washington 98021

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to, and remain with, the small business concern identified above with regard to the invention, entitled Chitinase Materials and Methods, by inventor(s) Patrick W. Gray,

described in

The specification filed herewith.

☒ Application Serial No. 08/663,618, filed June 14, 1996.

Patent No. _____ , issued _____.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: *Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).*

NAME: _____
ADDRESS: _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME: _____
ADDRESS: _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Judith Woods

TITLE OF PERSON OTHER THAN OWNER: Patent and Licensing Counsel

ADDRESS OF PERSON SIGNING: 22021 20th Avenue, S.E.,
Bothell, Washington, 98021

SIGNATURE: Judith A. Woods Date 8-13-96